Molecular analysis of genes acting in fruiting body development in basidiomycetes

Dissertation

In Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy (Ph.D.)

of the Faculty of Forest Sciences and Forest Ecology

Georg-August-Universität Göttingen

Submitted by

Prayook Srivilai

Born in Roi-Et, Thailand

Göttingen, 2006

D7

1st Examiner: Prof. Dr. Ursula Kües

2nd Examiner: Prof. Dr. Reiner Finkeldey

3rd Examiner: Prof. Dr. Stefan Schütz

Date of Oral Examination: 27.09.2006

Summary

Coprinopsis cinerea is an excellent model for studying fruiting body development in the higher basidiomycetes because it has a short life cycle, and grows and fruits on artificial media under laboratory conditions. The strain is accessible by both classical and molecular genetics. In addition, the *C. cinerea* genome has been released to the public making it an attractive species to study. At the present time, there are only a few species of mushrooms that are cultivated in farms on compost, straw and/or wood. By lack of knowledge, techniques for cultivation and fruiting development of other, highly priced species could not be developed up to now. Therefore, it is really necessary to study the growth and development of the higher basidiomycetes to support the increasing demand of the consumers of edible and also medicinal mushrooms. Furthermore, it is of interest to understand the growth and sexual development of wood decaying fungi that in humid tropical countries such as parts of Thailand will form mushrooms in and on houses, contributing to their destruction.

This thesis is focused on the action of genes in fruiting body development in basidiomycetes, using *C. cinerea* as a model. Development of fruiting bodies is regulated by both environmental (light, humidity, temperature and nutrients) and genetic factors of which the mating type genes are the major regulators. Sexual development of *C. cinerea* is regulated by the *A* and *B* mating type genes that encodes two types of homeodomian transcription factors (called HD1 and HD2) and pheromones and pheromone receptors, respectively. For fruiting body development, in the normal situation two sterile parental strains with haploid nuclei of different mating type (compatible monokaryons) have to fuse in order to under to give the secondary mycelium, the fertile dikaryon, on which the fruiting bodies can develop. Homokaryon AmutBmut is a versatile self-fertile mutant of the mating type genes (*Amut Bmut*) that does not need to mate with another strain in order to produce fruiting bodies. Thus, this strain is ideal for the production of mutants in the fruiting pathway. One of such mutants (strain 6-031) was studied in this work.

Co-isogenic strains with different wild type mating type specificities have been constructed from homokaryon AmutBmut by repetitive backcrosses against the self-fertile strain. The quality of the obtained co-isogenic strains (PS001-1 to PS001-15 and PS002-1 to PS002-24) was tested by molecular RAPD (Randomly Amplification Polymorphism DNA) markers. Co-isogenic strains have been used to follow up in a genetic cross (6-031 x PS001-1) mutant genes in fruiting body development. In this ways, three different mutations (*cfs1*, *mat* and *bad*) in the *AmutBmut* UV-mutant 6-031 could be separated. Gene *cfs1* for a cyclopropane fatty acid synthase acts in the initiation of fruiting body development. The other mutations in fruiting body development are in a gene for primordia maturation (*mat*) and in a gene defective in basidiospore production (*bad*).

Strain OU3-1 containing the *cfs1* mutation was obtained from the cross of 6-031 x PS1001-1 and served further in analyzing the *cfs1* function in fruiting development by transformation with the cloned *cfs1* wild type gene. The *cfs1* gene is found to be an essential gene for the fruiting body initiation in the basidiomycete *C. cinerea*.

The role of the *A* and *B* mating type genes in regulation of development were further studied by transformation of sub-cloned genes in suitable *C. cinerea* monokaryons as well as into an A*null* strain (NA2) lacking whole the *A* mating type locus. Both, mating type genes from *C. cinerea* as well as mating type genes from other basidiomycetes species were transformed. The existence of A*null* strain (NA2) demonstrates that these *A* mating type genes are not essential for growth. However, during colony growth of NA2 transformants containing a compatible pair of *A* mating type genes of different specificities repress asexual spore production (oidiation) in the dark. Some evidence is given that there might be also a reaction of single homeodomain transcription genes (*HD1*) on repression of oidiation.

Foreign homeodomain transcription factors from the basidiomycetes *Ustilago maydis*, *Schizophyllum commune* and *Coprinellus disseminatus* are able to interact with homeodomain transcription factors of *C. cinerea,* as judged from the specialized clamp cell production on monokaryotic transformants of *C. cinerea*. Production of clamp cells normally occur at the hyphal septa of the dikaryotic mycelium as mechanism to ensure the equal distribution of the two haploid parental nuclei present in the dikaryon. Clamp cell formation is long known by classical genetics to be under control of the *A* mating type genes. Further in this thesis, by transformation of *B* mating type–like genes from *C. disseminatus* into *C. cinerea* it could be shown that the foreign pheromone and pheromone receptor genes did act in clamp cell fusion as well as in regulation of fruiting body development.

As a further regulator of development, a dominant constitutively activated *ras* allele (*ras*^{Val19}), for a small GTPase active in different signalling pathways possibly including the *B* mating type pathway, has been transformed into different monokaryons of *C. cinerea.* Ras^{Val19} affected growth rates of monokaryons and dikaryons, tissue formation, during fruiting body development, and basidiospore production.

The two promoters of the *Sc3* and *Sc4* hydrophobin genes of *S. commune,* two developmentally regulated genes expressed in vegetative mycelium and fruiting bodies, respectively, were studied in activity in *C. cinerea*, making use of a laccase gene as a reporter. Expression from the two promoters were found in all development stages in *C. cinerea* but with a preference for the *Sc3* promoter in the vegetative mycelium and the *Sc4* promoter in the fruiting bodies, in accordance to their expression profiles in their original host.

Acknowledgements

This dissertation would not be completed without a good gesture from many persons who have contributed to this work. Firstly important, I would like to express my deepest sense of gratitude and sincerest appreciation to my supervisor, Professor Dr. Ursula Kües, for providing me with the good opportunity to work under her excellent supervision, for her guidance and tremendous encouragement very closely throughout the period of my Ph.D. study.

Secondly, my sincere appreciation and gratefulness are also expressed to Professor Dr. Reiner Finkeldey, Professor Dr. Stefan Schütz and Professor Dr. Alireza Kharazipour for serving as the dissertation committee, and also for their constructive and valuable comments.

My sincere gratitude is especially indebted to Dr. Timothy Y. James and Professor Dr. Rytas Vilgalys at Department of Biology, Duke University, Durham, North Carolina, for kindly providing me the plasmids used in this study, and very friendly encouraging supports and discussions.

Special thanks are also due to Dr. Patrik Hoegger Dr. Rosemarie Heyser and Dr. Andrzej Majcherczyk for teaching and advice me the molecular genetics, microscopy techniques and techniques of laboratory works.

I wish to express my warmest gratitude to the people in our '*Coprinopsis* group', namely Mojtaba Zomorrodi, Monica Navarro, Rajesh Velagapudi, Sreedhar Kilaru, Ravi Chandra Dwivedi, Wassana Chaisaena, Jhansi Kalyani Pemmasani, Sudhakar Peddireddi, Martin Rühl, Banyat Cherdshim, Ihtzaz Malik and Karin Lange for making the days in the lab enjoyable and productive, and also for their eternal helps and friendships.

My acknowledgement is also expressed to U. Kües Laboratory, Institute of Forest Botany, Faculty of Forest Sciences and Forest Ecology, Georg-August-University of Goettingen, for providing the excellent research facilities.

I recognize and gratefully acknowledge the Kor-Por-Bhor scholarship awarded by Mahasarakham University, Thailand for the financial support during the full-time period of my PhD study.

I owe my deepest gratitude and heartful thanks to my parents Mr. Prathana and Mrs. Boonyang Srivilai, my elder sister Mrs. Piyawan Dorndundit, my younger brother Mr. Prathan Srivilai for their eternal encouragement, support, and love which never be forgotten.

Finally, no more words to express my deepest and loving thanks belong to Ms. Panida Loutchanwoot for her love, support and patience, and for always believing in me.

Table of Contents

CHAPTER 1

Introduction

1.1. Mushrooms, problems, advantages and their applications

Life consists of three domains (superkingdoms), i.e. bacteria, archaea, and eukarya. The bacteria and archaea belong to prokaryotes; these two domains are believed to have diverged very early in the evolution of life. Eukarya then diverged from archaea, giving rise to the third domain, consisting of four kingdoms which are protista, fungi, plantae, and animalia. The fungi in the world are estimated at around 1.5 million (Hawksworth 1991). The number of the known species of fungi is about 69,000 of which 10,000 species are fleshy macro-fungi, including poisonous mushroom (Kendrick 1985). Fungal species constitute about 5% of the estimate total species in the world (Chang 1991). Fungi are eukaryotic, heterotrophic, absorptive organisms, have in most instances a branched and tubular body, and reproduce by production of spores (Kendrick 1985). A mushroom is most often used to describe the reproductive structure (fruiting body) of a fungus. The term “mushroom” can also mean any fungus which produces the fleshy fruiting body. By this definition, not all fungi are qualified as mushrooms (Arora 1986). Another definition of mushroom is a macro-fungus with a distinctive fruiting body that can be either grown under ground (hypogeous) or above ground (epigeous) (Hawksworth 1997; Chang and Miles 1993). In 1961, Atkinson defined a mushroom as one living organism belonging to the basidiomycetes. Toadstool is regarded as a synonymous term. An edible basidiomycete or, rarely ascomycete fungus had been defined as mushrooms by Gray (1967). Most mushrooms belong to basidiomycetes (singular: basidiomycete) which form fruiting bodies that contain the spore-bearing microscopic club-shaped cellular structures called basidia (singular: basidium). Some other mushrooms are defined as ascomycetes (singular: ascomycete) which produce spores (ascospores) inside the microscopic sac-like mother cells called asci (singular: ascus). The fruiting bodies of basidiomycete species and of ascomycete species are significantly different in their details. The main structures of the basidiomycete mushrooms consist of the cap with the hymenium (either calling gills or pores) and the stalk (Fig. 2). A protective covering structure found at fruiting bodies of some basidiomycets is called a veil that partially covers the gills during the young mature state of mushroom development, and breaks at maturation to form a ring on the stalk. In other species, the universal veil at first envelops the entire fruiting body, and then breaks to form a vulva (sack, collar, or series of concentric ring at the base of the stalk) (Arora 1986).

Based on their application and consumption, mushrooms can be divided into four categories, i.e. edible mushrooms, medicinal mushrooms, poisonous mushrooms, and non-defined mushrooms. Edible mushrooms (for example: *Agaricus bisporus, Auricularia auricula, Flammulina velutipes, Pleurotus spp.*, *Lentinus edodes*) and medicinal mushrooms (*Ganoderm spp., Tremella spp.*) are the sources of food and health components. They are easily grown on several substrates, i.e. straw-based compost, woods, and various wastes from agricultures (Stamets and Chilton 1989).

Figure 1. *Coprinopsis cinerea* (courtesy of Monica Navarro-González).

There are many white rot fungi such as *Schizophylum commune*, *Trametes versicolor*, *Pleurotus ostreatus, Dichomitus squalens* and *Phanerochaete chrysosporium* which decay wood, resulting in bleaching of the wood. White rot fungi need the wood texture which has enough moisture and oxygen, provides an optimum temperature and favorable pH, and contains chemical growth factors for fungi such as carbon and nitrogen compounds, vitamins and essential elements (Zabell and Morerell 1992). There are many types of wood that are suitable substrates for the white rot fungi such as alder and oak which are the most popular ones for use in constructions, paper industry and furniture (Blanchette 1984).

Wood is an organic material consisting of cellulose, hemicellulose, and lignin. Some fungi such as *T. versicolor*, *D. squalens* and *P. chrysosporium* have a higher efficiency to decay the wood than other white rot fungi. Wood weight loss in case of the formed species can be as high as around 39-65% after 12 weeks of incubation (Eriksson et al. 1990). The spores of white rot fungi germinate on the wet wood or a soft decayed wood which is undergoing decay by ascomycetes and some bacteria. The spore germinates to a mycelium which colonizes on the wood surface. Subsequently,

the hyphae penetrate into the wood, break down the wood cell walls and feed on the cell wall components over the time. The fungi destroy the wood with their appropriate properties. The mycelium finally may develop the fruiting bodies, in which sexual reproduction occurs. The sexual spores may either germinate to new mycelium occurring around the fruiting body area or may be distributed to other places by wind and insects (Tuno 1999; Sterling et al. 1999). Basidiomycetes normally release several enzymes to decay the wood, respectively the cellulose, hemicelluloses, and lignin components of the plant cell walls. White rots particularly secrete laccases and peroxidases. Brown rots decaying the cellulose secrete cellulases and hemicellulases (Bourbonnais et al. 1995; Eggert et al. 1997).

There are many basidiomycete species that can serve as nutritional and medicinal sources for human life. Many countries use mushrooms for daily consumption in addition to other vegetables. The edible mushrooms can grow and develop well in the forest which has low light amounts, high humidity and the appropriate temperature, conditions needed for fruiting. Alternatively, edible mushrooms may be cultivated by human, for example on straw or wood (Rühl and Kües 2006).

Mushrooms present a highly nutritious foodstuff that is rich in proteins that have a high amount of essential amino acids. For example, lysine and leucine, which are lacking in the cereal foods (Chang 1989). The fresh mushrooms contain large amounts of carbohydrate and fiber, ranging from 51-88% and 4-20%, respectively on the regular dry weight basis (Huang et al. 1989). The mushrooms are also a good source of various vitamins such as thiamine, riboflavin, niacin, biotin and ascorbic acid (Crisan and Sands 1978; Li and Chang 1982). Mushrooms also appear to be a good foodstuff for dieting due to the low crude fat content which varies from less than 1.0% to almost 10.0 % on a dry weight basis, and measures from all classes of the lipid components (Crisan and Sands 1978). The major mineral elements such as potassium, phosphorus, sodium, calcium, magnesium, and the minor minerals such as iron, copper, zinc are found in several edible mushrooms, but the amount of these mineral elements is relatively variable based on the species and strains of the mushrooms, and the substrates used for mushroom growing (Chang and Miles 1989; Buswell and Chang 1993). In Asian countries, especially Japan, Korea, and China, the people have used the mushrooms as one alternative of medicinal therapy to treat patients since 2000 years ago. At least 50 different medicinal mushrooms species have been used by the Chinese physicians (Beetz and Kustetudia 2004).

In 1990, 1995 and 2000, the world productions of the edible mushrooms were $1.76x10^6$, $2.05x10^6$, and $2.60x10^6$ metric tons, respectively. In 2005, the Food and Agriculture Organization of the United Nation (FAO) reported that the world production of the edible mushrooms was about $3.35x10^6$ metric tons (see http://www//faostat.fao.org/faostat). The edible mushrooms and medicinal

mushrooms have many benefits for the world's population. Nowadays, the wastes from agriculture, forestry and food industries are numerous and steadily increasing. Mushroom cultivation on such waste counteracts deterioration of the environment by the break down of lignocellulosic wastes which commonly act as pollutants, thus facilitating waste management (Chang 1993). In consequence of this, mushroom cultivation on these wastes can increase the values of agriculture and forestry wastes.

1.2. *Coprinopsis cinerea* and *Schizophyllum commune* are the genetic models of the homobasidiomycetes species (mushrooms) for studying fruiting body development

Coprinopsis cinerea and to a lesser extend *Schizophyllum commune* have served as the main model species for the homobasidiomycetes. *C. cinerea* grows on horse dung and straw, *S. commune* is a wood rotting fungus. These model organisms are extensively used to study particularly the fundamental biological principles such as metabolic, regulatory, and developmental pathways including fruiting body development and to investigate the relevant genes, and also evolution in order to provide and characterize insights and knowledge in comparison with other related organisms. On the long run, it is therefore hoped with studies on the model species to better understand also the biology of other species that can not as easily be grown in the lab as the model fungi and that unlike the two model species are not accessible by classical and molecular biological techniques (Kües 2000; Kothe 2001). By now, the genome of *Coprinopsis cinerea* is completely finished and released (see http://www.broad.mit.edu/annotation/fungi/*)*.

C. cinerea was until recently called *Coprinopsis cinereus* and belongs to the family of Agaricaceae (Redhead et al. 2001). The name of *C. cinereus* has been changed to *C. cinerea* due to the recent phylogeny analysis with molecular markers (ITS sequences) by the group of Vilgalys (Redhead et al. 2001), but some current publications still use the name *C. cinereus* (Riquelme et al. 2005). The common name of the fungus is ink cap mushroom and the natural substrate of this species is horse dung. In the past, mycologists isolated this fungus several times from the nature, and gave various different names for this fungus (Kües 2000). For example, Lewis and Day worked on the fungus under the name of *Coprinus lagopus* (Lewis 1961; Day and Anderson 1961), other names were *Coprinus delicatulus* Apinis, *Coprinus fimetarius* L. ex Fr., *Coprinus fimetarius* L. Fr. var. *macrorhizus* Pers. Ex. Fr., and *Coprinus macrorhizus* f. *microsporus* were in use (Liu 2001).

C. cinerea is an excellent model to study fruiting body development in the basidiomycetes. It is easily grown under the laboratory conditions utilizing artificial media, (for example: Yeast extract-Malt extract-Glucose: YMG/T medium, supplement Tryptophan) and its original natural substrate, i.e. horse dung (Walser et al. 2001). Most importantly for research, *C. cinerea* has the ability to complete its life cycle within two weeks (Walser et al. 2001; Kües 2000). In addition,

various molecular biological techniques have so far been developed in *C. cinerea* such as gene transformation (Binninger et al. 1987), REMI [Restriction Enzyme-Mediated Integration of (DNA)], a technique for mutant production by DNA transformation (Granado et al. 1997; Lui et al. 1999) and gene mapping with suitable molecular markers and chromosomal markers (Muraguchi et al. 2003; Zolan 1995; Zolan et al. 1994), as well as modern cytological techniques such as the Fluorescent In Situ Hybridization (FISH) (Li et al. 1999).

C. cinerea is a heterothallic basidiomycete, i.e. it has two types of mycelia in its life cycle, namely the sterile primary mycelium, the monokaryon and the secondary mycelium, the fertile dikaryon. Two monokaryons of different mating compatibility have to fuse in order to form the fertile dikaryon that can form the mushrooms (Fig. 2) (Casselton 1995; Kües 2000).

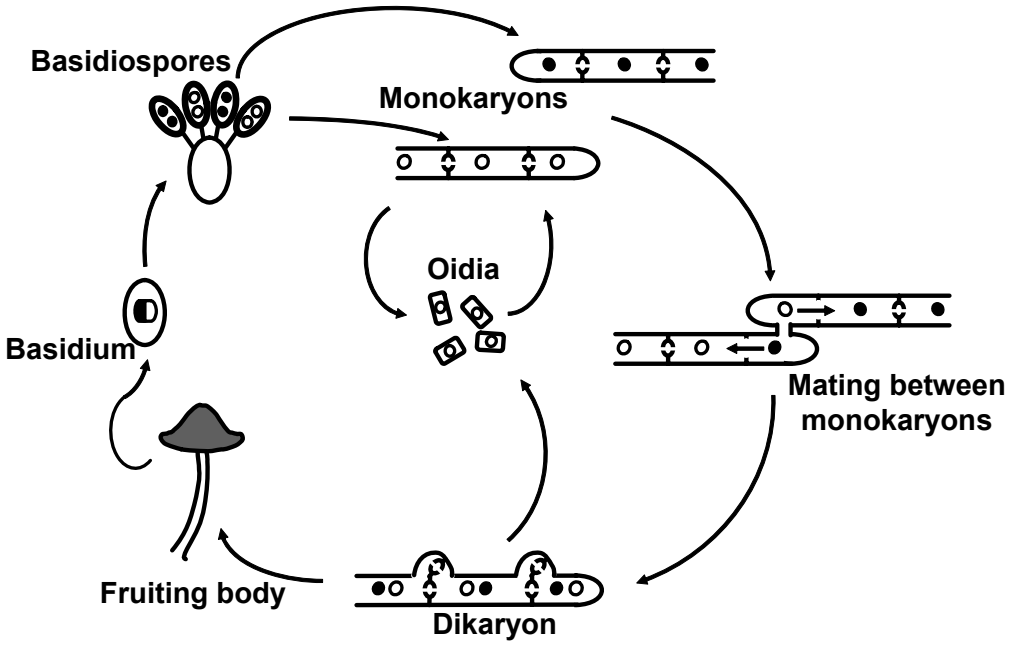


Figure 2. Life cycle of *Coprinopsis cinerea* (Kües 2000).

The life cycle of *C. cinerea* begins with the germination of haploid basidiospores under appropriate environmental conditions. The resulting monokaryons contain usually one individual haploid nucleus, defined mating type specificity per hyphal cell or sometimes also two (Kües 2002). The monokaryon has simple septa and constitutively forms vegetative small spores (oidia) in the aerial mycelium, and in older age, large inflated single cells (chlamydospores) on agar-air interfaces (Kües et al. 1998; Polak et al. 1998). Furthermore, monokaryons may develop aerial and submerged sclerotia, which are multicellular structures that have a brown coloured pigment in their outer cell layers (rind). Sclerotia serve as resting structures to overcome situations with adverse environmental conditions (Moore 1979, 1981; Elhiti and Butler 1979).

Mating between two compatible monokaryons gives rise to a dikaryon, the secondary mycelium which has two different haploid nuclei per hyphal cell, one from each of the two different parents. Clamp cells are formed above the hyphal septa. Clamp cells serve in the correct distribution of the two types of parental nuclei in cell division. Usually at a later stage, close to the clamp cells, hyphal side branches will be formed (Kües 2000; Casselton and Economou 1985). On the dikaryon under fruiting conditions (temperature: 26-28 °C, humidity: 80-90% and 12-hours light/12-hours dark), hyphal knots develop by aggregation of the mycelia to initiate the fruiting body development. Mycelial aggregation can be divided into two levels, the primary hyphal knot formed in the dark and the secondary hyphal knot that requires light. The primary hyphal knots form by intense localized hyphal branching and packing of several short generative hyphae. Under light induction, some of the primary hyphal knots will grow into the fluffy spherical mycelial bodies called the secondary hyphal knots, which have a size around 1 to 2 mm. Without light induction, the primary hyphal knots will develop into globose, small multicellular, persistent sclerotia of approximately 250 µm in diameter (Moore 1981; Kües 2000; Kües et al. 1998; Kües and Liu 2000, Fig 3).

Inside the small packing of secondary hyphal knots, cell differentiation occurs, whilst the structures (now called primordia) grow in size up to 1 cm. Stipe and cap tissues are formed under control of further light signals. When primordia development is finished, within specialized cells (basidia) karyogamy and meiosis occur by a light induction. With induction of karyogamy, the primordia are transformed into the immature fruiting bodies. The clusters of parallel-orientated hyphae that form the mushroom stipe elongate, whilst the cells of the apical prosenchymal tissue that form the pileus (young mushroom cap) inflate during the maturation of fruiting body development (Kamada and Tasura 1993; Muraguchi and Kamada 1998; Kües 2000). The development of the primordia to the mature fruiting body comprises at least four major processes, i.e. basidiospore formation, stipe elongation, cap expansion and, after fruiting body maturation, the cap autolysis (Kües 2000; Kamada 2002). During basidiospore formation by a budding-type process on the basidia, each one haploid nucleus that arose by meiosis is migrating from the basidium into a spore. Subsequently, the basidiospore stains black by melanin incorporation into the spore cell walls. Basidiospore formation is finished when the cap is fully opened. Only a few hours after, the cap autolysis occurs thereby releasing the spores (Kües 2000).

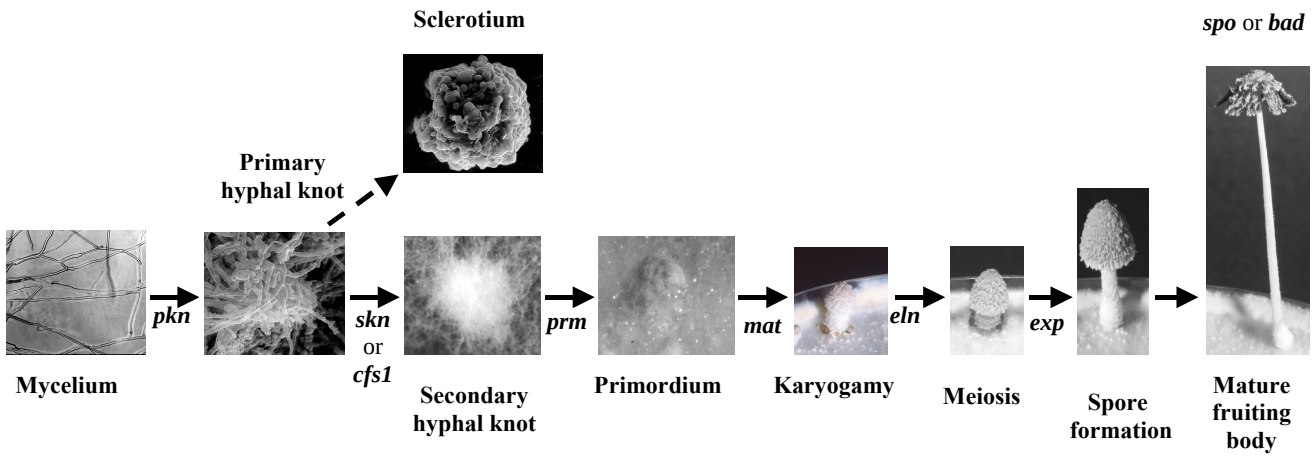


Figure 3. Fruiting body development of *Coprinopsis cinerea* [adapted and modified from the previous publication of Boulianne et al. (2000)]. Multiple mutants have been generated in the past that are blocked at different stages in the fruiting body development stages (Kües and Granado, unpublished). Names of groups of defective genes are indicated in figure. Mutants unable to form primary hyphal knot (*pkn*) show defects in the aggregation of the mycelium. Clones which show a defect in the transition of the primary hyphal knots to the secondary hyphal knots are known as *skn* (secondary hyphal knot). Mutants which have mutations in development of the primordium are called *prm* mutants (for primordium maturation). Mutants which show defects in the maturation of the fruiting body are called *mat* mutants (for maturation). *eln* mutations (stipe elongation) influence the elongation of the stipe, *cap* mutations influence the caps expansion (*exp*). Mutants that have defects in basidiospore formation have white caps due to lack of the black coloured basidiospores *bad* or *spo* (Kües 2000; Kothe 2003).

S. commune as a wood rotting fungus is another mushroom model to study fruiting body development in the basidiomycetes (Raper 1973; Wessels 1968). In contrast to the quickly autolysing fruiting bodies of *C. cinerea,* the fan-shaped mushroom of *S. commune* are long lasting. Dehydration is the way of storage of the fruiting body of *S. commune* for years. Further growth of the fruiting body and further spore formation can be simply induced by remoistening (Wessels 1965). *S. commune* is also a heterothallic species that is widely distributed throughout the temperate and tropical zones, and commonly found on wood of deciduous trees, but it also occurs as a weak parasite on a wide range of woody plants (Raper 1966; Wessels 1993; Peddireddi et al. 2005). Also this fungus easily grows under laboratory conditions on artificial medium and has a short life cycle (Wessels 1965), following the criteria for the qualifications of organismic models. Unfortunately for this fungus a completed sequence DNA genome is not yet available, but the sequencing is in performance (see http://www.broad.mit.edu/annotation/fungi/fgi/). *S. commune* performs in sexual reproduction comparably to *C. cinerea*, and has alternating monokaryons and dikaryons in its life cycle (Kothe 2001; Wessels 1993).

Also in *S. commune*, dikaryotic mycelia produce the fruiting bodies. Primordia are formed by the extensive proliferation of closely packed vegetative cells (Raper 1966), which further develop to the sporulating fruiting body at the expense of the surrounding mycelia due to transfer of nutrients. Inside each basidium on the surface of the fruiting body's gills, there are two parental nuclei of the dikaryotic mycelium. These will first fuse and in subsequent meiosis divide into four haploid gametes nuclei that will migrate into the four basidiospores. Basidiospores of *S. commune* are not stained. As in *C. cinerea*, sexual reproduction in *S. commune* is controlled by different mating type specificities that are developed by the genes of the two different *A* and *B* mating type loci (classically known as *A* and *B* factors). The two fungi are therefore tetrapolar. A tetrapolar fungus gives rise in meiosis to four different mating type specificities (*AxBx*, *AyBx*, *AxBy* and *AyBy*; X and Y referring to different mating type specificities). Upon germination of spores, only those monokaryons can fuse to a fertile dikaryon that are different at both mating type loci, i.e. *AxBx* can react with *AyBy*, and *AxBy* with *AyBx* (Raper 1966; Kothe 2001).

Figure 4. *Schizophyllum commune* (courtesy of Sudhakar Peddireddi).

Upon fusion of two mating compatible monokaryons, the *A* mating type gene of *S. commune* encoding homeodomain transcription factors control the clamp formation, clamp septation, and synchronization of nuclear division as do the *A* mating type genes in *C. cinerea*. The *B* mating type locus of *S. commune* contains subloci, namely $B\alpha$ and $B\beta$ loci, and each encode pheromones and a pheromone receptor (Vaillancourt et al. 1997; Wendland et al. 1995). These genes control nuclear migration during mating of two compatible monokaryons, sub-apical peg formation at hyphal septa prior to clamp cell fusion, and the clamp cell fusion with the peg (Badalyan et al. 2004; Schubert et al. 2006).

1.3. The mating type system in the basidiomycete *C. cinerea*

In *C. cinerea* as in *S. commune*, the *A* mating type locus is located on the first of the totally 13 chromosomes, adjacent to the mitochondria intermediate peptidase (*MIP*) gene and the para-aminobenzoic acid synthase (*pab1)* (Giasson et al. 1989; Mutasa 1990; James et al. 2004a). The *A* mating type locus is composed of two subloci, namely the *Aα* and the *Aβ* sublocus, both of which have multiple alleles. The *Aα* locus contains either an *a1* allele or an *a2* allele or alleles of both genes which together are called the *A* mating type gene a pair. The *a1* gene encodes an HD1 protein, and the *a2* encodes an HD2 protein. HD1 and HD2 refer to distinct homeodomain sequences (specific DNA binding domains) within the encoded transcription factors. The *Aβ* locus contains three different gene pairs, i.e. *b1* and *b2*, *c2* and *c1*, and *d1* and *d2.* Also these encode two the different types of HD proteins (Kües 2000; Kües et al. 1994a; Kües et al. 1994b; Pardo et al. 1995). In sexual development, at least one HD1 and one HD2 protein from different parental original have to interact to give an active transcription factor complex. It is further important to note that only proteins from allelic gene pairs interact but not proteins from different gene pairs or proteins from a same gene pair. Several studies utilizing DNA transformation revealed that only a single compatible HD1-HD2 heterodimer from *A* mating type genes is needed to trigger for example clamp cell formation (Kües and Casselton 1992; Casselton and Kües 1994; Kües et al. 1994a; Kües et al. 2002; Pardo et al. 1996). Furthermore, the active heterodimers from *A* mating type genes of different parental origin suppress oidia production on the aerial mycelium on the dikaryon under dark conditions (Kües et al. 1998; Kertesz-Chaloupková et al. 1998; Kües et al. 2002; more details on the *A* mating type genes are given in Chapter 5).

In *C. cinerea*, the *B* mating type locus is located on the second chromosome or tenth chromosome, depending on which of available gene maps it is referred to, either the classical map established by North (1990) or the molecular marker map established by Muraguchi et al. (2003). The *B* mating type locus has three independent gene subfamilies which are called sub-family 1, sub-family 2, and sub-family 3. Each subfamily consists of one gene encoding a pheromone receptor (*rcb1, rcb2,* and *rcb3,* respectively) and two genes encoding pheromones (*phb1-1* and *phb1-2*, *phb2-1* and *phb2-2*, and *phb3-1* and *phb3-2*, respectively) (Halsall et al. 2000; Kües 2000; O'Shea et al. 1998; Riquelme et al. 2005). Based again on transformation studies, it was concluded that for sexual reproduction a pheromone receptor has to interact with pheromones that come from an allelic subfamily of genes from a nucleus of a compatible mating type (more details on the *B* mating type genes are given in Chapter 5).

1.4. Technical reasons for choosing *C. cinerea* as a model of studies

In this study, the model fungus *C. cinerea* is used to give more insight into sexual development of basidiomycetes, for reasons that complete fruiting body development in *C. cinerea* is fast (5 days) and moreover, in its successive steps it is synchronized to the day/night rhythm, unlike that of *S. commune*. In addition, the structure of fruiting bodies and the course of development are much better described in *C. cinerea* than in *S. commune* (Walser et al. 2003). Most importantly, hundreds of mutants are available in fruiting of *C. cinerea* due to the fact that we have self-compatible homokaryons with defects in both mating type loci (*Amut Bmut* strain) that allow fruiting without mating to another strain and, therefore, after mutagenesis of the haploid uninucleate (oidia), to easily find not only dominant but also recessive mutations in fruiting body development (further information in chapter 2). Transformation studies showed so far only in *C. cinerea*, that the *A* mating type genes control fruiting body initiation and that the *B* mating type genes control karyogamy, and thereby fruiting body maturation. In addition, the *B* mating type genes enhance the fruiting inducing effect of the *A* mating type genes (Tymon et al. 1992; Kües et al. 1998, 2002).

C. cinerea by far has the best transformation system of all basidiomycetes that up to date can be transformed, giving several hundreds to over one thousand transformants in a single experiment (Bininger et al. 1987; Granado et al. 1997). As mentioned already above, these enable easy studies of testing functions of cloned mating type genes in *C. cinerea*. As a special, a knock-out mutant of the *A* mating type locus exists (Pardo et al. 1995; Polak 1999), in which the *B* mating type genes can be analyzed without any *A* gene and, from the *A* locus, *HD1* and *HD2* genes can be analyzed independently from each other. Transformation of heterologous mating type genes into *C. cinerea* has been shown before that such genes can induce sexual development in this species (Challen et al. 1993, further information present in chapter 5). Thus, *C. cinerea* can also be used in testing functions of mating type genes of other species.

Other genes acting, respectively suspected to act in fruiting, had been cloned before from *C. cinerea* [*cfs1* for a cyclopropane fatty acid synthase (Lui 2001); *ras* for a GTPase of the Ras type (Bottoli 2001)], that were available for further studies in this study. An efficient reporter system for studying expression from specific promoters has recently been established in *C. cinerea* (Kilaru et al. 2006; Kilaru 2006). This system allowed us with promoters from *S. commune* hydrophobin genes expressed in different developmental stage of the fungus [the *SC3* and *SC4* promoters that are expressed in vegetative mycelia and the fruiting bodies (Mulder and Wessels 1986; Wessels et al. 1987), to test whether heterologous genes from specific developmental stages are expressed in *C. cinerea* the same developmental manner than in *S. commune*. At the beginning of this thesis, the only technical disadvantage of using *C. cinerea* compared to *S. commune* was a lack of co-isogenic

strains of different mating types. In *S. commune,* such co-isogenic strains were constructed already in 1960ties (Raper 1996; Wessels 1997).

1.5. Aims of the study

The present study aimed at molecular analysis of genes acting in fruiting body development of the basidiomycetes. In particular, the elucidation of the following issues was therefore addressed:

1. To construct co-isogenenic monokaryotic strains of the self-compatible *C. cinerea* homokaryon AmutBmut with different wild type mating type specificities for the purpose to easily analyze genes in fruiting body development by classical genetics (Chapter 2 to 3).
2. To investigate an essential gene for fruiting body initiation in the basidiomycete *C. cinerea* (*cfs1*) cloned by Lui (2001) that is homologous to bacterial cyclopropane fatty acid synthase genes, by DNA transformation in a suitable homokaryotic mutant of the mating type loci obtained from genetic crosses (Chapter 4).
3. To further analyze the function of homologous mating type genes in *C. cinerea* as well as of heterologous mating type genes from the wood rotting basidiomycetes *Coprinellus disseminatus*, *S. commune*, and *Ustilago maydis* (Chapter 5 to 7).
4. To study promoters from different developmental stages from the wood rotting basidiomycete *S. commune* in development of *C. cinerea* (Chapter 9).
5. To study the function of a mutant *ras* gene (ras^{Val19} whose product is constitutively activated) in vegetative growth and fruiting body development in *C. cinerea* (Chapter 8).
6. In Chapter 10, the obtained results will be discussed and set in relation.

1.6. References

Adrio, J. L., and A. L. Demain, 2003 Fungal biotechnology. Int. Microbiol. **6:** 191-199.

Arora, D., 1986 *Mushrooms Demystified*. Ten Speed Press, Berkeley, California.

Badalyan, S. M., E. Polak, M. Aebi, and U. Kües, 2004 Role of peg formation in clamp cell fusion of homobasidiomycete fungi. J. Basic Microbiol. **44:** 167-177.

Beetz, A., and M. Kustetudia, 2004 *Mushroom Cultivation and Marketing* (http://www Attar.ncat.org.).

Binninger, D. M., C. Skrzynia, P. J. Pukkila, and L. A. Casselton, 1987 DNA-mediated transformation of the basidiomycete *Coprinus cinereus*. EMBO J. **6:** 835-840.

Blanchette, R., 1984 Screening wood decayed by white rot fungi for preferentail lignin degradation. Appl. Env. Microbiol. **48:** 647-653.

Buswell, J., and S. T. Chang, 1993 Edible mushrooms: attributes and applications, pp. 297-324 in *Genetics and Breeding of Edible mushrooms*, edited by S. T. Chang, J. Buswell, and P. G. Miles. Gordon and Breach Science Publishers, The Netherlands.

Burnett, J. H., 1976 *Fundamentals of Mycology*. Edward Arnold, Ltd., London.

Casselton, L. A., 1995 Genetics of *Coprinus*, pp. 35-48 in *The Mycota, Vol. II., Genetics and Biotechnology*, edited by U. Kües. Springer-Verlag KG, Berlin, Germany.

Casselton, L. A., and A. Econoumou, 1985 Dikaryon formation, pp. 213-229 in *Developmental Biology of Higher Fungi*, edited by D. Moore, L. A. Casselton, D. A. Wood and J. C. Frankland. Cambridge University Press, Cambridge.

Chang, S. T., 1980 Mushrooms as human food. Bioscience **30:** 399-401.

Chang, S. T., 1991 Mushroom and mushroom biology, pp. 1-13 in *Genetics and Breeding of Edible Mushrooms*, edited by S. T. Chang, J. Buswell and P. G. Miles. Gordon and Breach Science Publishers, The Netherlands.

Chang, S. T., 1993 Mushroom biology: The impact on mushroom production and mushroom products, pp. 3-20 in *Mushroom Biology and Mushroom Products*, edited by S. T. Chang, J. A. Buswell and S. W. Chiu. The Chinese University Press, Hong Kong.

Chang, S. T., and P. G. Miles, 1989 *Edible Mushrooms and Their Cultivation*. CRC Press, Boca Raton, Florida.

Chang, S. T., and P. G. Miles, 1991 Recent trends in world production of cultivated mushrooms. Mushroom J. **503:**15-18.

Crisan, E. V., and A. Sands, 1978 Nutritional value, pp.137-165 in *The Biology and Cultivation of Edible Mushroom*, edited by S. T. Chang and W. A. Hayes. Academic Press, New York.

Day, P. R., 1963 Structure of a mating type factor in *Coprinus lagopus*-Wild alleles. Genet. Res. **4:** 323-325.

Eriksson, K. E. L, R. A. Blanchette, and P. Ander, 1990 *Microbial and Enzymatic Degradation of Wood and Wood Components*. Springer-Verlag KG, Berlin.

Granado, J. D., K. Kertesz-Chaloupkova, M. Aebi, and U. Kües, 1997 Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. Mol. Gen. Genet. **256:** 28-36.

Gray, P., 1967 Activity-passivity. J. Am. Psychoanal. Assoc. **15:** 709-728.

Halsall, J. R., M. J. Milner, and L. A. Casselton, 2000 Three subfamilies of pheromone and receptor genes generate multiple *B* mating specificities in the mushroom *Coprinus cinereus*. Genetics, **154:** 1115-1123.

Hawksworth, D. L., 1991 The fungal dimension of diversity: magnitude, significance, and conservation. Mycol. Res. **95:** 641-655.

Hawksworth, D. L., 1997 Fungi and biodiversity: international incentives. Microbiologia **13:** 221-226.

Huang, B. H, K. H. Yung, and S. T. Chang, 1989 Fatty acid composition of *Volvariella volvacea* and other edible mushroom. Mushroom Sci. **12:** 533-540.

James, T. Y., U. Kües, S. A. Rehner, and R. Vilgalys, 2004a Evolution of the gene encoding mitochondrial intermediate peptidase and its cosegregation with the *A* mating-type locus of mushroom fungi. Fungal Genet. Biol. **41:** 381-390.

James, T. Y., S. R. Liou, and R. Vilgalys, 2004b The genetic structure and diversity of the A and B mating-type genes from the tropical oyster mushroom, *Pleurotus djamor*. Fungal Genet. Biol. **41:** 813-825.

James, T. Y., P. Srivilai, U. Kües, and R. Vilgalys, 2006 Evolution of the bipolar mating system of the mushroom *Coprinellus disseminatus* from its tetrapolar ancestors involves loss of mating-type-specific pheromone receptor function. Genetics **172:** 1877-1891.

Kamada, T., 2002 Molecular genetics of sexual development in the mushroom *Coprinus cinereus*. Bioessays **24:** 449-459.

Kendrick, B., 1985 *The Fifth Kingdom*. Focus Information Group, Newburyport.

Kendrick, B., 1979 Mitospores in basidiomycetes, pp. 473-545 in *The Whole Fungus*, edited by B. Kendrick. National Museum of Canada, Ottawa.

Kertesz-Chaloupková, K., P. J. Walser, J. D. Granado, M. Aebi, and U. Kües, 1998 Blue light overrides repression of asexual sporulation by mating type genes in the basidiomycete *Coprinus cinereus*. Fungal Genet. Biol. **23:** 95-109.

Kilaru, S., 2006 *Identification of Fungal Multi-Copper Oxidase Gene Families: Overexpression and Characterization of Coprinopsis cinerea Laccase for Applications in Biotechnology*. Ph.D thesis, Georg-August-University of Göttingen, Göttingen, Germany.

Kilaru, S., P. J. Hoegger, A. Majcherczyk, C. Burns, K. Shishido *et al.*, 2006 Expression of laccase gene *lcc1* in *Coprinopsis cinerea* under control of various basidiomycetous promoters. Appl. Microbiol. Biotechnol. **71:** 200-210.

Kothe, E., 2001 Mating-type genes for basidiomycete strain improvement in mushroom farming. Appl. Microbiol. Biotechnol. **56:** 602-612.

Kües, U., 2000 Life history and developmental processes in the basidiomycete *Coprinus cinereus*. Microbiol. Mol. Biol. Rev. **64:** 316-353.

Kües, U., and L. A. Casselton, 1992 Homeodomains and regulation of sexual development in basidiomycetes. Trends Genet. **8:** 154-155.

Kües, U., and L. A. Casselton, 1993 The Origin of Multiple Mating Types in Mushrooms. J. Cell Sci. **104:** 227-230.

Kües, U., and Y. Liu, 2000 Fruiting body production in Basidiomycetes. Appl. Microbiol. Biotechnol. **54:** 141-152.

Kües, U., R. N. Asante-Owusu, E. S. Mutasa, A. M. Tymon, E. H. Pardo *et al.*, 1994a Two classes of homeodomain proteins specify the multiple a mating types of the mushroom *Coprinus cinereus*. Plant Cell **6:** 1467-1475.

Kües, U., B. Göttgens, R. Stratmann, W. V. Richardson, S. F. O'Shea *et al.*, 1994b A chimeric homeodomain protein causes self-compatibility and constitutive sexual development in the mushroom *Coprinus cinereus*. EMBO J. **13:** 4054-4059.

Kües, U., A. M. Tymon, W. V. Richardson, G. May, P. T. Gieser *et al.*, 1994c *A* mating-type factors of *Coprinus cinereus* have variable numbers of specificity genes encoding two classes of homeodomain proteins. Mol. Gen. Genet. **245:** 45-52.

Kües, U., J. D. Granado, R. Hermann, R. P. Boulianne, K. Kertesz-Chaloupkova *et al.*, 1998 The A mating type and blue light regulate all known differentiation processes in the basidiomycete *Coprinus cinereus*. Mol. Gen. Genet. **260:** 81-91.

Kües, U., T. Y. James, R. Vilgalys, and M. P. Challen, 2001 The chromosomal region containing *pab-1*, *mip,* and the *A* mating type locus of the secondarily homothallic homobasidiomycete *Coprinus bilanatus*. Curr. Genet. **39:** 16-24.

Kües, U., P. J. Walser, M. J. Klaus, and M. Aebi, 2002 Influence of activated *A* and *B* mating-type pathways on developmental processes in the basidiomycete *Coprinus cinereus*. Mol. Genet. Genomics **268:** 262-271.

Li, G. S. F., and S. T. Chang, 1982 Nutritive value of *Vovariella volvacea*, pp.199-219 in *Tropical Mushrooms-Biological Nature and Cultivation Methods*, edited by S. T. Chang and T. H. Quimio. The Chinese University Press, Hong Kong.

Moore, D., 1981 Developmental genetics of *Coprinus cinereus*-genetic-evidence that carpophores and sclerotia share a common pathway of initiation. Curr. Genet. **3:** 145-150.

Moore, D., 1998 *Fungal Morphogenesis*. Cambridge University Press, Cambridge.

Moore, D., M. M. Y. Elhiti, and R. D. Butler, 1979 Morphogenesis of the carpophore of *Coprinus cinereus*. New Phytol. **83:** 377-382.

Muraguchi, H., Y. Ito, T. Kamada, and S. O. Yanagi, 2003 A linkage map of the basidiomycete *Coprinus cinereus* based on random amplified polymorphic DNAs and restriction fragment length polymorphisms. Fungal Genet. Biol. **40:** 93-102.

Mutasa, E. S., A. M. Tymon, B. Göttgens, F. M. Mellon, P. F. R. Little *et al.*, 1990 Molecular organization of an *A*-mating type factor of the Bbasidiomycete fungus *Coprinus cinereus*. Curr. Genet. **18:** 223-229.

North, J., 1990 Linkage map of *Coprinus cinereus* Schaeff. Ex Fr. S.F.Gray, Ink cap. N=12, pp. 3.70-3.81 in *Genetic Maps: Locus Maps of Complex Genomes*, Ed. 5., edited by S. J. O'Brien. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

O'Shea, S. F., P. T. Chaure, J. R. Halsall, N. S. Olesnicky, A. Leibbrandt *et al.*, 1998 A large pheromone and receptor gene complex determines multiple B mating type specificities in *Coprinus cinereus*. Genetics **148:** 1081-1090.

Pardo, E. H., S. F. O'Shea, and L. A. Casselton, 1996 Multiple versions of the *A* mating type locus of *Coprinus cinereus* are generated by three paralogous pairs of multiallelic homeobox genes. Genetics **144:** 87-94.

Polak, E., 1999 *Asexual Sporulation in the Basidiomycete Coprinus cinereus*. PhD thesis, ETH Zurich, Zurich, Switzerland.

Polak, E., R. Hermann, U. Kües, and M. Aebi, 1997 Asexual sporulation in *Coprinus cinereus*: structure and development of oidiophores and oidia in an amut bmut homokaryon. Fungal Genet. Biol. **22:** 112-126.

Peddireddi S., R. Velagapudi, P. J. Hoegger, A. Majcherczyk, A. Naumann *et al.*, 2005. Multiple hydrophobin genes in mushrooms in *Proceedings of the Genetics and Cellular Biology of Basidiomycetes VI.*, edited by A. G. Pissabaro and L. Ramírez. Spain Universidad Pública de Navarra, Pamplona, Spain, in press.

Raper, C. A., and J. R. Raper, 1973 Mutational analysis of a regulatory gene for morphogenesis in *Schizophyllum*. Proc. Natl. Acad. Sci. USA **70:** 1427-1431.

Raper, J. R., 1966 *Genetics of Sexuality in Higher Fungi*. Ronald Press, New York.

Redhead, S. A., R. Vilgalys, J. M. Moncalvo, J. Johnson, and J. S. Hopple, 2001 *Coprinus* Pers. and the disposition of *Coprinus species* sensu lato. Taxon **50:** 203-241.

Riquelme, M., M. P. Challen, L. A. Casselton, and A. J. Brown, 2005 The origin of multiple *B* mating specificities in *Coprinus cinereus*. Genetics **170:** 1105-1119.

Rühl, M, and U. Kües 2006 Mushroom production in *Wood production, Wood technology and Biotechnological Impacts*, edited by U. Kües. Universitätsverlag Göttingen, Göttingen, Germany, in press.

Schubert, D., M. Raudaskoski, N. Knabe, and E. Kothe, 2006 Ras GTPase-activating protein Gap1 of the homobasidiomycete *Schizophyllum commune* regulates hyphal growth orientation and sexual development. Eukaryot. Cell **5:** 683-695.

Sterling, M., C. Rogers, and E. Levetion, 1999 An evaluation of two methods used for microscopic analysis of air bone fungal spore conditions from the Burkard Spore Trap. Aerobiologia **15:** 9-18.

Stewart, G. R., and D. Moore, 1974 Activities of glutamate-dehydrogenases during mycelial growth and sporophore development in *Coprinus lagopus* (sensu Lewis). J. Gen. Microbiol. **83:** 73-81.

Swamy, S., I. Uno, and T. Ishikawa, 1984 Morphogenetic effects of mutations at the *A* and *B* incompatibility factors in *Coprinus cinereus*. J. Gen. Microbiol. **130:** 3219-3224.

Tuno, N., 1999 Insect feeding on spores f a bracket fungus *Elfvingia applanata* (parst. Ganodermataceae, Aphylophorales. Ecol. Res. **14:** 7-103.

Vaillancourt, L. J., M. Raudaskoski, C. A. Specht, and C. A. Raper, 1997 Multiple genes encoding pheromones and a pheromone receptor define the *Bβ*1 mating-type specificity in *Schizophyllum commune*. Genetics **146:** 541-551.

van Wetter, M. A., H. A. Wösten, J. H. Sietsma, and J. G. Wessels, 2000 Hydrophobin gene expression affects hyphal wall composition in *Schizophyllum commune*. Fungal Genet. Biol. **31:** 99-104.

van Wetter, M. A., H. A. Wösten, and J. G. Wessels, 2000 SC3 and SC4 hydrophobins have distinct roles in formation of aerial structures in dikaryons of *Schizophyllum commune*. Mol. Microbiol. **36:** 201-210.

Waters, H., R. D. Butler, and D. Moore, 1975 Structure of aerial and submerged sclerotia of *Coprinus lagopus*. New Phytol. **74:** 199-208.

Wendland, J., L. J. Vaillancourt, J. Hegner, K. B. Lengeler, K. J. Laddison, *et al.*, 1995 The mating-type locus *Bα*1 of *Schizophyllum commune* contains a pheromone receptor gene and putative pheromone genes. EMBO J. **14:** 271-5278.

Zolan, M. E., 1995 Chromosome-length polymorphism in fungi. Microbiol. Rev. **59:** 686-698.

Zolan, M. E., N. K. Heyler, and N. Y. Stassen, 1994 Inheritance of chromosome-length polymorphisms in *Coprinus cinereus*. Genetics **137:** 87-94.

CHAPTER 2

Genetic analysis of *Coprinopsis cinerea* mutants with defects in fruiting body development

Srivilai P., Chaisaena W., and U. Kües

Published in: VI Genetic and Cellular Biology of Basidiomycetes (G. Pisabarro and L. Ramírez, eds.), Universidad Pública de Navarra, Pamplona, Spain, *in press*.

This manuscript is a preliminary compilation of classical genetic work preformed in my thesis. The work on the mutant B-1918 was done by Wassana Chaisaena under my direction. Prior to acceptance for publication, the manuscript was peer-reviewed by experts in fungal genetics.

2.1. Abstract

Few genes have so far been cloned and characterized in fruiting of the heterothallic mushroom *Coprinopsis cinerea*. Fruiting body development normally occurs on the dikaryon. However, the binucleate state of the mycelium hinders easy access of genes. Self-compatible mutants with defects in the mating type pathways can form fruiting bodies without prior fusion to another strain. Uninucleate haploid oidia of such mutants can easily be mutagenized and germinated mycelia tested for defects in fruiting. Mutants can be produced from oidia by classical techniques such as UV treatment or by modern REMI (restriction enzyme-mediated integration) mutagenesis via transformation. Such mutants of self-compatible strains have now been successfully appointed in cloning genes acting in sexual development. Co-isogenic strains of compatible mating types support in genetic characterisation of the mutants.

2.2. Introduction

2.2.1. The wild-type life cycle

Coprinopsis cinerea (formerly called *Coprinus cinereus*; Redhead et al. 2001) is an excellent model to study fruiting body development in the basidiomycetes. It easily grows in the laboratory and completes its life cycle (Fig. 1, Kües 2000) within two weeks on its natural substrate horse dung as well as on artificial substrates on yeast extract-malt extract-glucose basis (Walser et al. 2001). The life cycle of the heterothallic *C. cinerea* starts with germination of basidiospores that contain one type of haploid nuclei. The resulting primary mycelia are called monokaryons. They have simple septa and one or sometimes two genetic identical haploid nuclei in their hyphal compartments. Monokaryons constitutively produce in high numbers single-celled, uninucleate haploid mitotic spores (oidia) on specialized aerial structures, the oidiophores (Polak et al. 1997, 2001; Kües et al. 2002a; Fischer and Kües 2003). As long as nutrients are available, monokaryons can grow indefinitely. In nature, however, dikaryons are prevailing because as soon as they meet, monokaryons of different mating type will fuse to form these secondary mycelia. The dikaryon is characterized by a vigorous mycelium of usually faster growth compared to the parental monokaryons. It has two distinct haploid nuclei in the hyphal compartments (Iwasa et al. 1998) and clamp cells at the hyphal septa (Buller 1933; Badalyan et al. 2004). Under specific environmental conditions, fruiting bodies are formed on the dikaryon (Moore 1998; Kües 2000; Wösten and Wessels 2005).

Oidia production on the dikaryon is repressed in the dark. In light, oidia are produced but in much lower numbers than on the monokaryons (Kertesz-Chaloupková et al. 1998; Kües et al. 2002b). The uninucleate haploid oidia are short-lived and serve in distribution of the species to new substrate and as fertilizing agent in fusion with monokaryons of different mating type (Brodie 1931; Kemp 1977; Kües 2002; Fischer and Kües 2005).

Light is also needed for induction of fruiting on the dikaryon (Tsusué 1969; Morimoto and Oda 1973; Lu 1974). However, fruiting occurs only under high humidity when nutrients are low and temperatures are in the range of 25-28°C (Madelin 1956; Walser 1997).

Tissue formation within the primordium and fruiting body maturation including karyogamy, meisois and basidiospore production is adapted to the daily dark/light rhythm (Lu 1974; Moore et al. 1979). For the process to correctly proceed, further to initiation, light signals as well as distinct dark phases are required at specific points of development (Lu 1974, 2002; Kamada et al. 1978). Once all tissues in the primordium are established, light induced karyogamy parallels induction of fruiting body maturation. Meiosis directly follows karyogamy in the basidia. Stipes elongate and caps open with proceeding meiosis and basidiospore production and maturation. The mature

fruiting body appears black by the colour of the melanized cell walls of the ripe basidiospores. The fruiting body is short-lived. Within a few hours, it quickly undergoes autolysis for spore liberation (Moore 1998; Kües 2000; Kamada 2002).

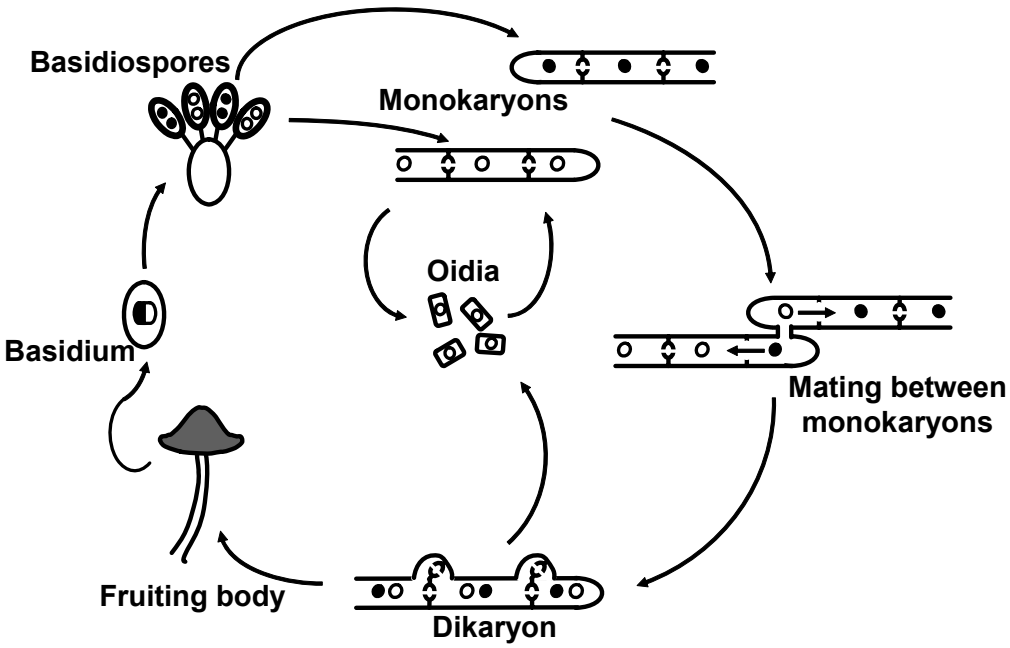


Figure 1. Life cycle of *Coprinopsis cinerea* (modified from Kües 2000). Filled and open circles indicate haploid nuclei of different mating type, a larger half filled-half open circle in the basidium the diploid nucleus obtained by fusion of two haploid nuclei of different mating type. For simplicity of the diagram, oidia production is only shown for one monokaryon.

2.2.2. Mutants in fruiting body development on the dikaryon

Fruiting bodies normally develop on the dikaryon, which hinders genetic analysis of the process. Upon mutagenesis of the dikaryon, one would expect to only detect dominant mutations and a very low total number of mutants in screenings for defects in fruiting. For detection of recessive genes, principally two different nuclei would have to be mutagenized and this in the same cell and in the same gene. Such double mutants should be hard to find, particularly when using for mutagenesis mycelium with many dikaryotic cells in which many nuclei will be left non-mutagenized. Takemaru and Kamada used macerated mycelium of a *C. cinerea* dikaryon in UV mutagenesis and chemical mutagenesis with NG (*N*-methly-*N'*-nitro-*N*-nitroso-guanidine). Surprisingly, they found abnormalities in fruiting body development in frequencies of over 10% of tested clones, in total 1,594 developmental variants amongst 10,641 tested isolates (Takemaru and Kamada 1969, 1970, 1972). Takemaru and Kamada (1972) suggested three causes for the high amount of variants in their studies: i) influence of factors other than genes, ii) mutations in dominant genes, and iii) easy access of fruiting genes in mutagenesis. Later on, Moore (1981)

pointed out, that there are already many recessive genetic defects in fruiting present within the natural genetic pool of *C. cinerea*. For example, he observed in his wild-type strain collection defective alleles in four different genes acting in fruiting body initiation. In addition, we found that in the Okayama 7 strain (see http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/) a natural defect in an essential fruiting initiation gene due to the insertion of a transposon (unpublished). In support of Moore's view, other natural defects in sexual development have been described in tissue formation of the primordium (Muraguchi and Kamada 1998), in formation of basidiospores (Pukkila 1993; Kües et al. 2002b), in activation of fruiting in homokaryons (Uno and Ishikawa 1971; Murata et al. 1998a, 1998b; Muraguchi et al. 1999), and in the process of nuclear exchange during mating (May and Taylor 1988). Furthermore, during crosses, new defects spontaneously arise (see below).

Few genes in fruiting have been cloned and analysed since Takemaru and Kamada did their mutagenesis study on the dikaryon. A respectable reason for the low number of cloned genes in fruiting is certainly the normally required dikaryotic state that requests an enormous work load and clever combinations of classical and molecular approaches when wanting to identify a gene (Muraguchi and Kamada 1998, 2000).

As a first gene, the pileus-specific *ich1* gene (for *ichijiku*, the Japanese word for fig) was cloned by first identifying the chromosome it locates on and then complementing the spontaneous recessive *ich1* mutation through transformation of a chromosome-specific library into an *ich1* defective monokaryon followed by crosses of transformants to another compatible *ich1* strain. *ich1* mutants fail to differentiate pileus tissue at the apex of the primordial stipe. Lack of pileus tissue causes a dent in the normally egg-shaped primordial giving the structure a fig-like shape. The abnormal *ich1* primordia rupture during stipe maturation and basidiospores are not formed, unless the defect is complemented by transformation with the wild-type gene. Ich1 is a large protein of 1353 amino acids that contains a potential nuclear targeting signal and has therefore been suggested to act within the nucleus (Muraguchi and Kamada 1998). Moreover, the protein has in its N-terminal half a potential S-adenosyl-methionine (SAM) binding domain similar to known O-methyltransferases (Kües 2000). Dominant genes might be obtained in analogous strategies but by transformation into a wild-type monokaryon prior to mating of transformants to a compatible wild-type strain. *eln2* (*elongationless 2*) is a constitutively expressed gene that encodes a novel type of microsomal cytochrome P450 enzyme termed CYP502. A dominant *eln2* mutation (originally identified in a self-compatible background, see below) affects stipe tissue formation in the primordia and results in dumpy fruiting bodies with short stipes. The mutant gene was found by altered phenotype on the dikaryon after transforming a wild-type monokaryon and crossing to another strain (Muraguchi and Kamada 2000).

2.2.3. Self-compatible mutants in studying fruiting body development

Fruiting body development has been shown to be controlled by the mating type genes (Tymon et al. 1992; Kües et al. 1998, 2002b). The genes at the *A* mating locus, encoding homeodomain transcription factors (Hiscock and Kües 1999; Casselton and Challen 2005) control light-induced initiation of fruiting. However, development is arrested after tissues formation in the primordial and before karyogamy occurs in the basidia (Tymon et al. 1992; Kües et al. 1998). The genes at the *B* mating type locus, encoding pheromones and pheromone receptors, respectively (Kothe 2001; Casselton and Challen 2005), support the *A* mating type genes in their function in initiating fruiting body development. Primordia are formed in higher numbers and at an earlier time when both pathways are active. Moreover, after completion of tissue formation in the primordia, development continues leading to mature fruiting bodies. This suggests that the *B* mating type genes are required for induction of karyogamy (Kües et al. 2002b).

Consistent with the above results from monokaryons transformed with heterologous mating type genes, mutants with defects in the two mating type loci are self-compatible and have a simplified life-cycle (Fig. 2). Such homokaryotic *Amut Bmut* strains form fruiting bodies with basidiospores without mating to another strain (Swamy et al. 1984; Boulianne et al. 2000; Fig. 3 and Fig. 4). These basidiospores germinate into the self-compatible vigorous mycelia of dikaryon-like appearance (Fig. 3, left photo). The mycelium of *Amut Bmut* homokaryons has clamp cells at most septa (Fig. 4, photo at the left), but only one type of haploid nuclei in its hyphal cells. In submerged medium, there are mostly two nuclei per hyphal cell and in there aerial mycelium, there is often only one nucleus in a hyphal cell (Swamy et al. 1984; Polak et al. 1997). *Amut Bmut* homokaryons produce uninucleate haploid oidia that again grow into self-compatible mycelia (Swamy et al. 1984; Fig. 3, 2nd photo from left). However, oidia are not constitutively produced in the aerial mycelium as in monokaryons, but asexual spore formation needs illumination as in dikaryons (Polak et al. 1997; Kertesz-Chaloupková et al. 1998). Also like in dikaryons, light induces fruiting on the established mycelium when nutrients are exhausted (Walser et al. 2003; Kües et al. 2004). Upon primordia formation, karyogamy of genetic identical nuclei occurs in the basidia. Meiosis follows and, during fruiting body maturation, the production of four identical basidiospores (Swamy et al. 1984; Kanda et al. 1989a; Fig. 3 photos to the right, Fig. 4).

Nowadays, *Amut Bmut* homokaryons [either the original homokaryon AmutBmut from Swamy et al. (1984) or *A43mut*, *B43mut* homokaryon 326 created by Pukkila (1993, 1996) from homokaryon AmutBmut through repeated backcrosses to monokaryon 218] are in most instances used in mutant production (Kanda and Ishikawa 1986; Kanda et al. 1989a, 1989b; Chiu and Moore 1990; Pukkila 1994; Granado et al. 1997; Cummings et al. 1999; Inada et al. 2001; Arima et al.

2004; Kües et al., unpublished; see below). Several genes have been cloned from UV and REMI mutants of such self-compatible homokaryons (Celerin et al. 2000; Inada et al. 2001; Arima et al. 2004; Liu et al. submitted). Amongst cloned functions acting in mushroom formation is a gene *cfs1* for a cyclopropane fatty acid synthase (Liu 2001; Liu et al., submitted) and a gene *eln3* for putative membrane protein with a general glycosyltransferase domain (Arima et al. 2004).

Alternatively to *Amut Bmut* homokaryons, self-compatible strains with defects in the mating type pathways downstream of the mating type genes might be used for mutant production and gene recovery, e.g. the Cop5D mutant being defective in a gene *pcc1* for an HMG box transcription factor acting in the *A* mating type pathway (Murata et al. 1998a, 1998b; Muraguchi et al. 1999; Muraguchi and Kamada 2000). The dominant *eln2* gene is an example of a gene detected through mutagenesis of homokaryon Cop5D (Muraguchi and Kamada 2000, see below).

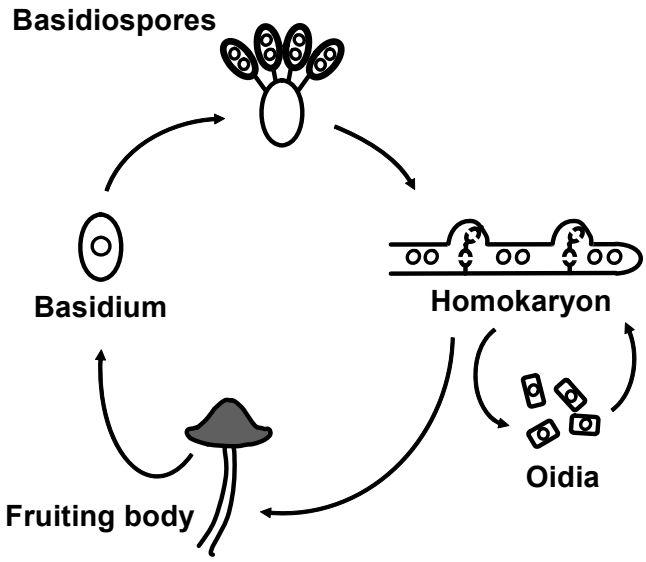


Figure 2. Life cycle of the self-compatible *Coprinopsis cinerea* homokaryon AmutBmut that is defective at both mating type loci (Swamy et al. 1984). Small open circles indicate haploid nuclei, a large open circle the homozygous diploid nucleus in the basidium.

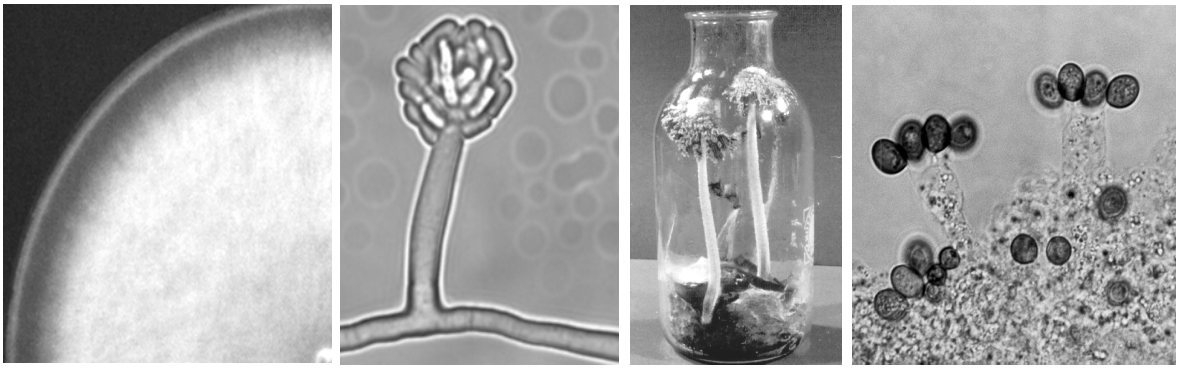


Figure 3. *Coprinopsis cinerea* strain AmutBmut (*A43mut*, *B43mut*, *pab1-1*), a homokaryon originally isolated by Swamy et al. (1984). From left to right: vegetative mycelium, an oidiophore produced upon light induction, mushrooms on horse dung and basidia with each four basidiospores. Photos are of courtesy of Yi Liu, Eline Polak, Markus Aebi and Jose Granado.

2.3. Materials and methods

C. cinerea homokaryon AmutBmut (*A43mut*, *B43mut*, *pab1-1*; Swamy et al. 1984; May et al. 1991) was used in mutant production of UV mutant 6-031 (*A43mut*, *B43mut*, *pab1-1*, *skn1*, *mat*, *bad*) and REMI mutant B-1918 (*A43mut*, *B43mut*, *pab1-1*, *dst3*), (Liu et al. 1999 and submitted, Chaisaena et al. unpublished). Monokaryons JV6 (*A42*, *B42*), 218 (*A3*, *B1*, *trp-1.1, 1.6*, *bad*) and PS001-1 (*A42*, *B42*; co-isogenic to homokaryon AmutBmut) were used in crosses (Kertesz-Chaloupková et al. 1998; Srivilai et al., in preparation). R1428 (*A8*, *B7*, *dst1-2*) was kindly supplied by T. Kamada. Growth conditions and all genetic methods are given in Walser et al. (2001).

2.4. Results and Discussion

2.4.1. Mutant production with self-compatible *Coprinopsis cinerea* homokaryons

The *pab1-1*-auxotrophic homokaryon AmutBmut (Fig. 3, Fig. 4) carrying the mating type alleles *A43mut* and *B43mut* (Swamy et al. 1984; May et al. 1991) has repeatedly been used in the past to create mutants in fruiting body development including meiosis and basidiospore production (Kanda and Ishikawa 1986; Kanda et al. 1989a, 1989b; Chiu and Moore 1990; Pukkila 1994; Granado et al. 1997; Kües et al., unpublished). Mutagenesis is easy since the haploid oidia can be used both for classical UV mutagenesis (Kanda et al. 1989a, 1989b) as well as for transformation in modern REMI (restriction enzyme- mediated integration) mutagenesis (Granado et al. 1997). Upon light illumination, oidia production on *A43mut*, *B43mut* homokaryons is abundant with numbers of up to 10^9 spores per plate (Kertesz-Chaloupková et al. 1998). Established UV and REMI mutagenesis protocols of oidia from homokaryon are given by Walser et al. (2001). Both UV and REMI mutagenesis has been performed by our group with homokaryon AmutBmut. About 10,000 mutants were screened for behaviour in fruiting. More than 1,200 mutants were detected that were affected in mycelial growth, asexual sporulation and/or fruiting body development (Granado et al. 1997; Polak 1999; Kües et al., unpublished).

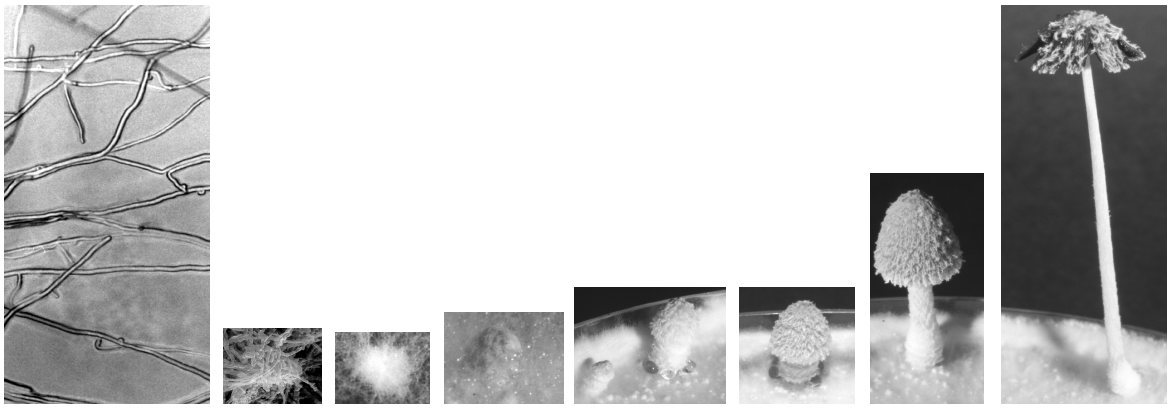


Figure 4. Stages in fruiting body development of *Coprinopsis cinerea* homokaryon AmutBmut (not to scale; adapted with alterations from Boulianne et al. 2000). Within the established mycelium (note the clamp cells at some of the septa in the left photo), loose aggregates (primary hyphal knots) form in the dark by localized intense formation of short hyphal branches with restricted tip growth. Upon reception of a light signal, hyphae aggregate into the compact secondary hyphal knots, in which tissue formation occurs. Correct tissue formation in the primordia needs changing day-night rhythms. Karyogamy in the basidia is induced by a light signal and directly followed by meiosis and basidiospore formation. Parallel to meiosis and basidiospore formation, the stipe elongates and the cap opens. Since it is a highly synchronized process, the developmental stages within the basidia can be predicted by the outer appearance of stages in mushroom development (further details can be found in Lu 1974, 2002; Moore et al. 1979; Kües 2000; Liu 2001; Walser et al. 2003; Kües et al. 2004).

Using the scheme in Fig. 4, mutants were classified into three major groups with each several hundreds of mutants. The first group comprises defects in fruiting body initiation (block in primary hyphal knot formation and block in secondary hyphal knot formation), the second defects in primordia development up to the stage of karyogamy and the third defects in fruiting body maturation including defects in meiosis and basidiospore formation, respectively (Kües et al. unpublished). The frequencies of mutant production in homokaryon AmutBmut is thus as high as in the original mutant screens with dikaryons performed by Takemaru and Kamada (1972) and as high as in screens with other self-compatible homokaryons of *C. cinerea* (Cummings et al. 1999; Muraguchi et al. 1999).

2.4.2. Gene cloning with mutants of self-compatible *Coprinopsis cinerea* homokaryons

A gene in secondary hyphal knot formation (*skn1*) has recently been cloned by direct complementation of the AmutBmut UV mutant 6-031 (Liu et al., submitted). For transformations, an AmutBmut genomic library was used present in a cosmid carrying the wild-type *C. cinerea pab1* gene (Bottoli et al. 1999). *pab1* complements the *pab1-1* auxotrophy of homokaryon AmutBmut and encodes a para-aminobenzoic acid synthase (James et al. 2002).

Whilst the defect in the early step of fruiting was complemented in the original UV mutant, complete fruiting body development was not achieved (Liu et al., submitted). Crosses of mutant 6-031 with unrelated monokaryons suggested further mutations in later stages of fruiting to be present in the mutant. However, the results of crosses were difficult to interpret because of large progeny fractions were unable to initiate fruiting or development arrested at different stages in development. Large quantities of progeny from parallel crosses between homokaryon AmutBmut and the same monokaryons also failed to initiate fruiting whilst others initiated but did not complete fruiting. Therefore, failure of initiation and completion of fruiting body development in the progenies of crosses of mutant 6-031 and monokaryons were in many instances likely not due to the *skn1* defect (Liu et al. 1999; Liu 2001; Srivilai et al., in preparation). Sequencing of the DNA fragment complementing the defect in fruiting body initiation in the *skn1* mutant identified the wild-type *cfs1* gene for a potential cyclopropane fatty acid synthase (Liu et al., submitted).

Handling REMI mutants might also not be as easy as originally thought. REMI mutants can carry more than one insertion (Granado et al. 1997; Liu et al. 1999), requesting separation by crosses prior to cloning the interesting insertion by plasmid rescue or PCR-mediated approaches (for techniques of inserted DNA recovery see Cummings et al. 1999 and Walser et al. 2001). However, genetic analysis of progeny of AmutBmut REMI mutants with unrelated monokaryons can be as difficult as with the UV mutants (Liu et al. 1999). As another hindrance found by other researchers (Inada et al. 2001), REMI insertions in some instances are unlinked to the mutant phenotype.

In conclusion, careful genetic analysis is advisable for both UV and REMI mutants before starting cloning genes. So far, this was difficult to perform for mutants of homokaryon AmutBmut.

2.4.3. Creating monokaryons with different mating type specificities that are co-isogenic to *Coprinopsis cinerea* homokaryon AmutBmut

In the past, few attempts have been made to create co-isogenic monokaryons in *C. cinerea* that distinguish just by mating types (Pukkila 1993). Therefore, we crossed *A43mut*, *B43mut* homokaryon AmutBmut to monokaryon JV6 with an *A42, B42* mating type and to monokaryon 218 with an *A3*, *B1* mating type. In the first generations, the fruiting abilities of *A43mut*, *B43mut* strains were very poor. In contrast, in higher filial generations of backcrosses to homokaryon AmutBmut, the mycelial appearance of clones in the progenies resembled that of homokaryon AmutBmut and the fruiting abilities raised above 90% of all *A43mut*, *B43mut* clones (Srivilai et al., in preparation). As a positive side effect from the first filial generation of the cross with monokaryon 218, we isolated a spontaneous *A43mut*, *B43mut* mutant with dumpy mushrooms (UFO1, see Fig. 5).

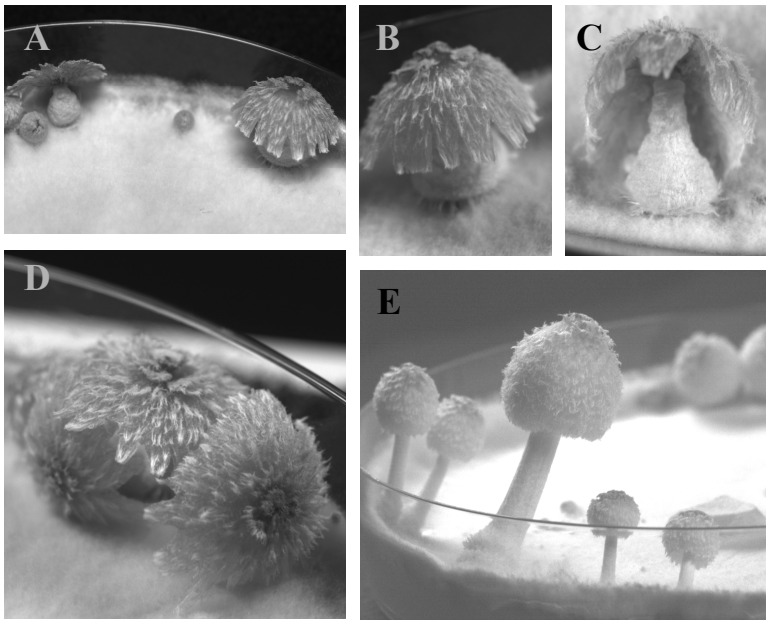


Figure 5. Mutant UFO1 (*A43mut*, *B43mut*, *pab1-1*) forms dumpy mushrooms due to a semi-dominant defect in stipe elongation (*eln*) gene (**A-D**). Moreover, it has no basidiospores due to a *bad* defect in basidiospore formation obtained from monokaryon 218 (Pukkila 1993; Kües et al. 2002). **E**. The UFO1 x 218 dikaryon forms medium-sized mushrooms suggesting that the *eln* defect in mutant UFO1 is semi-dominant. Mushrooms have white caps by lack of basidiospore production due to the homozygotous *bad* situation in the dikaryon.

2.4.4. Co-isogenic, mating compatible monokaryons in crosses with the secondary hyphal knot UV mutant 6-031 of *Coprinospis cinerea* homokaryon AmutBmut

When crossing the *skn1* mutant 6-031 with the compatible co-isogenic monokaryons, it was easy to separate the *skn1* mutation from a *mat* mutation (primordia maturation) and a *bad* mutation (basidiospore formation) that were also present in the mutant. Patterns of inheritance of such crosses were clear (Liu et al., submitted; Srivilai et al., in preparation; Fig. 6).

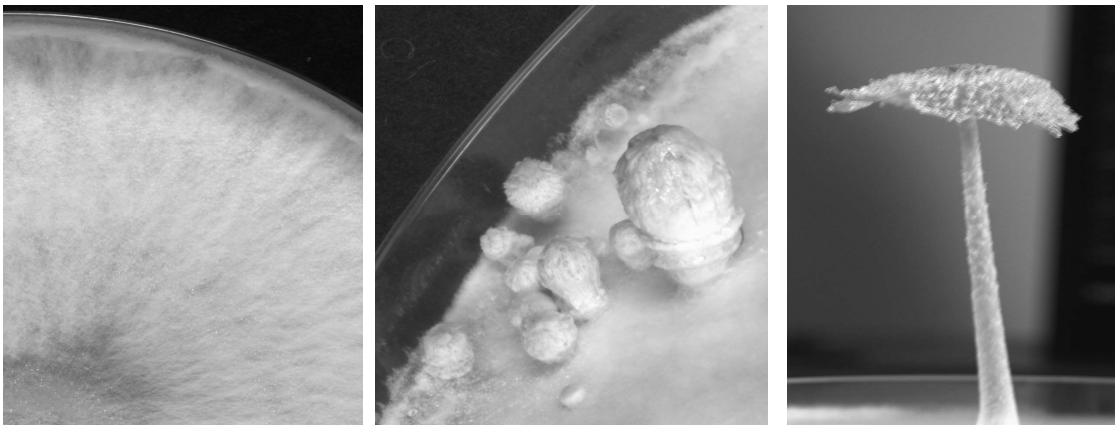


Figure 6. Phenotypes of *A43mut*, *B43mut* progeny of UV mutant 6-031 crossed with the compatible co-isogenic monokaryon PS001-1. From left to right: a *skn1* clone unable to initiate fruiting, a *mat* clone unable to produce mature fruiting bodies and a *bad* clone forming white mushrooms without spores.

2.4.5. AmutBmut REMI mutant B-1918

B-1918 is a REMI mutant of homokaryon AmutBmut that forms in light “etiolated stipes”, also called “dark stipes” (Liu et al. 1999; Fig. 7). In the wild-type, etiolated stipes appear when a strain did not receive enough light (Lu 1974). T. Kamada kindly supplied monokaryon R1428 (*A8*, *B7*, *dst1-2*) that carries a recessive defect in a light receptor and causes in dikaryons etiolated stipe formation in light when present in both type of haploid nuclei (Yuki et al. 2003). A B-1918 x R1428 dikaryon forms mature fruiting bodies (Fig. 7), indicating that the two mutants do not carry the same *dst* defect. In the homokaryotic *A43mut*, *B43mut* situation, the *dst1-2* gene gives in light rise to etiolated stipes (Fig. 7).

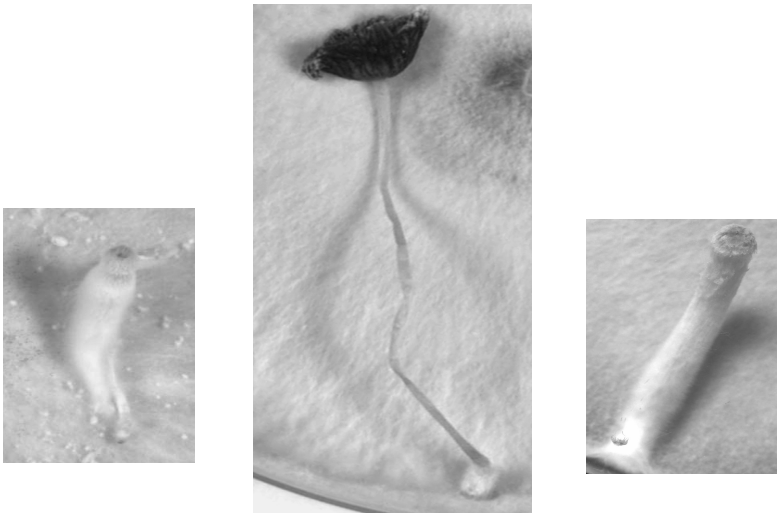


Figure 7. Mutant B-1918 forms etiolated stipes under dark condition (left). In crosses with R1428, mature mushrooms arise on the dikaryon. *A43mut*, *B43mut*, *dst1-2* clones from the progeny AmutBmut x R1428 form etiolated stipes in the light and do not give rise to mature fruiting bodies.

2.5. Conclusions

The self-compatible *C. cinerea* homokaryon AmutBmut has been used for producing mutants in fruiting body development. In the past, genetic analysis of these mutants was difficult to perform by lack of co-isogenic compatible monokaryons. We now have co-isogenic strains that allow fast genetic access of mutants and clear-cut interpretations of inheritance of mutant genes in progenies of crosses with AmutBmut mutants.

2.6. Acknowledgements

PS and WC hold PhD scholarships of the Mahasarakham University (Thailand) and the University of Technology Lanna Phitsanulok Campus (Thailand), respectively. Our laboratory is supported by the Deutsche Bundesstiftung Umwelt (DBU).

2.7. References

Arima, T., M. Yamamoto, A. Hirata, S. Kawano, and T. Kamada, 2004 The *eln3* gene involved in fruiting body morphogenesis of *Coprinus cinereus* encodes a putative membrane protein with a general glycosyltransferase domain. Fungal Genet. Biol. **41:** 805-812.

Badalyan, S. M., E. Polak, M. Aebi, and U. Kües, 2004 Role of peg formation in clamp cell fusion of homobasidiomycete fungi. J. Basic Microbiol. **44:** 167-177.

Bottoli, A. P. F., K. Kertesz-Chaloupková, R. P. Boulianne, J. D. Granado, M. Aebi *et al.*, 1999 Rapid isolation of genes from an indexed genomic library of *C. cinereus* in a novel *pab1*$^{+}$ cosmid. J. Microbiol. Meth. **35:** 129-141.

Boulianne, R. P., Y. Liu, M. Aebi, B. C. Lu, and U. Kües, 2000 Fruiting body development in *Coprinus cinereus*: regulated expression of two galactins secreted by a non-classical pathway. Microbiology **146:** 1841-1853.

Brodie, H. J., 1931 The oidia of *Coprinus lagopus* and their relation with insects. Ann. Bot. **45:** 315-344.

Buller, A. H. R., 1933 *Researches on Fungi.* Hafner Publishing Co., New York.

Casselton, L. A., and M. P. Challen, 2005 The mating type-genes of the basidiomycetes in *The Mycota*, Vol. I, Ed.2., edited by U. Kües and R. Fischer. Springer, Heidelberg, Germany, in press.

Celerin, M., S. T. Merino, J. E. Stone, A. M. Menzie, and M. E. Zolan, 2000 Multiple roles of Spo11 in meiotic chromosome behavior. EMBO J. **19:** 2739-2750.

Chiu, S.W., and D. Moore, 1990 A mechanism for gill pattern formation in *Coprinus cinereus*. Mycol. Res. **94:** 320-326.

Cummings, W. J., M. Celerin, J. Crodian, L. K. Brunick, and M. E. Zolan, 1999 Insertional mutagenesis in *Coprinus cinereus*: use of a dominant selectable marker to generate tagged, sporulation-defective mutants. Curr. Genet. **36:** 371-382.

Fischer, R., and U. Kües, 2003 Developmental processes in filamentous fungi, pp. 41-118 in *Genomics of Plants and Fungi*, edited by R. A. Prade and H. J. Bohnert. Marcel Dekker, New York.

Fischer, R., and U. Kües, 2005 Asexual sporulation in mycelial fungi in *The Mycota*, Vol. I, Ed.2., edited by U. Kües and R. Fischer. Springer, Heidelberg, Germany, in press.

Granado, J. D., K. Kertesz-Chaloupková, M. Aebi, and U. Kües, 1997 Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. Mol. Gen. Genet. **256:** 28-36.

Hiscock, S. J., and U. Kües, 1999. Cellular and molecular mechanisms of sexual incompatibility in plants and fungi. Int. Rev. Cytol. **193:** 165-295.

Inada, K., Y. Morimoto, T. Arima, Y. Murata, and T. Kamada, 2001 The *clp1* gene of the mushroom *Coprinus cinereus* is essential for *A*-reglulated sexual development. Genetics **157:** 133-140.

Iwasa, M., S. Tanabe, and T. Kamada, 1998 The two nuclei in the dikaryon of the homobasidiomycete *Coprinus cinereus* change position after each conjugate division. Fungal Genet. Biol. **23:**110-116.

James, T. Y., R. P. Boulianne, A. P. F. Bottoli, J. D. Granado, M. Aebi *et al.*, 2002 The *pab1* gene of *Coprinus cinereus* encodes a bifunctional protein for para-aminobenzoic acid (PABA) synthesis: implications for the evolution of fused PABA synthases. J. Basic Microbiol. **42:** 91-103.

Kamada, T., 2002 Molecular genetics of sexual development in the mushroom *Coprinus cinereus*. BioEssays **24:** 449-459.

Kamada, T., R. Kurita, and T. Takemaru, 1978 Effects of light on basidiocarp maturation in *Coprinus macrorhizus*. Plant Cell Physiol. **19:** 263-275.

Kanda, T., and T. Ishikawa, 1986 Isolation of recessive developmental mutants in *Coprinus cinereus.* J. Gen. Appl. Microbiol. **32:** 541-543.

Kanda, T., A. Goto, K. Sawa, H. Arakawa, Y. Yasuda *et al.*, 1989 Isolation and characterization of recessive sporeless mutants in the basidiomycete *Coprinus cinereus*. Mol. Gen. Genet. **216:** 526-529.

Kemp, R. F. O., 1977 Oidial homing and the taxonomy and speciation of basidiomycetes with special reference to the genus *Coprinus*, pp. 259-276 in *The Species Concept in Hymenomycetes*, edited by H. Clémençon. Cramer-Verlag, Vaduz, Liechtenstein.

Kertesz-Chaloupková, K., P. J. Walser, J. D. Granado, M. Aebi, and U. Kües, 1998 Blue light overrides repression of asexual sporulation by mating type genes in the basidiomycete *Coprinus cinereus*. Fungal Genet. Biol. **23:** 95-109.

Kothe, E., 2001 Mating-type genes for basidiomycete strain improvement in mushroom farming. Appl. Microbiol. Biotechnol. **56:** 602-612.

Kües, U., 2000 Life history and developmental processes in the basidiomycete *Coprinus cinereus*. Microbiol. Mol. Biol. Rev. **64:** 316-353.

Kües, U., 2002 Sexuelle and asexuelle Fortpflanzung bei einem Pilz mit 12000 Geschlechtern. Vierteljahrsschrift der Naturforschenden Gesellschaft in Zürich, **147:** 23-34.

Kües, U., J. D. Granado, R. Hermann, R. P. Boulianne, K. Kertesz-Chaloupková *et al.*, 1998 The *A* mating type and blue light regulate all known differentiation processes in the basidiomycete *Coprinus cinereus*. Mol. Gen. Genet. **260:** 81-91.

Kües, U., E. Polak, A. P. F. Bottoli, M. Hollenstein, P. J. Walser *et al.*, 2002a. Vegetative development in *Coprinus cinereus*, pp. 133-164 in *Molecular Biology of Fungal Development*, edited by H. D. Osiewacz. Marcel Dekker, Basel.

Kües, U., P. J. Walser, M. J. Klaus, and M. Aebi, 2002b Influence of activated *A* and *B* mating type pathways on developmental processes in the basidiomycete *Coprinus cinereus*. Mol. Gen. Genome. **268:** 262-271.

Kües, U., M. Künzler, A. P. F. Bottoli, P. J. Walser, J. D. Granado *et al.*, 2004 Mushroom development in higher basidiomycetes; Implications for human and animal health, pp. 431-470 in *Fungi in Human and Animal*, edited by R. K. S. Kushwaha. Scientific Publishers, Jodhpur, India.

Liu, Y., 2001 *Fruiting Body Initiation in the Basidiomycete Coprinus cinereus*. Ph.D. thesis, ETH Zurich, Zurich, Switzerland.

Liu, Y., J. D. Granado, E. Polak, and U. Kües, 1999 Crosses with *Amut Bmut* homokaryons of *Coprinus cinereus*. Fungal Genet. Newsl. **46:** 16-20.

Lu, B. C., 1974 Meiosis in *Coprinus*: VI. The control of the initiation of meiosis. Can. J. Genet. Cytol. **16:** 115-164.

Lu, B. C., N. Gallo, and U. Kües, 2003 White-cap mutants and meiotic apoptosis in the fungus *Coprinus cinereus*. Fungal Genet. Biol. **39:** 82-93.

Madelin, M. F., 1956 Studies on the nutrition of *Coprinus lagopus* Ann. Bot. **20:** 307-330.

May, G., and J. W. Taylor, 1988 Patterns of mating and mitochondrial DNA inheritance in the agaric Basidiomycete *Coprinus cinereus.* Genetics **118:** 213-220.

May, G., L. Le Chevanton, and P. J. Pukkila, 1991 Molecular analysis of the *Coprinus cinereus* mating type *A* factor demonstrated an unexpectedly complex structure. Genetics **128:** 529-538.

Moore, D., 1981 Developmental genetics of *Coprinus cinereus*: Genetic evidence that carpophores and sclerotia share a common pathway of initiation. Curr. Genet. **3:** 145-150.

Moore, D., 1998 *Fungal Morphogenesis*. Cambridge University Press, Cambridge.

Moore, D., M. M. Y. Elhiti, and R. D. Butler, 1979 Morphologenesis of the carpophore of *Coprinus cinereus*. New Phytol. **83:** 377-382.

Morimoto, N., and Y. Oda, 1973 Effects of light on fruit-body formation in a basidiomycete, *Coprinus macrorhizus*. Plant Cell Physiol. **14:** 217-225.

Muraguchi, H., and T. Kamada, 1998 The *ich1* gene of the mushroom *Coprinus cinereus* is essential for pileus formation in fruiting. Development **125:** 3133-3141.

Muraguchi, H., and T. Kamada, 2000 A mutation in *eln2* gene encoding a cytochrome P450 of *Coprinus ciner*eus affects mushroom morphogenesis. Fungal Genet. Biol. **29:** 49-59

Muraguchi, H., T. Takemaru, and T. Kamada, 1999 Isolation and characterization of developmental variants in fruiting using a homokaryotic fruiting strain of *Coprinus cinereus*. Mycoscience **40:** 227-235.

Murata, Y., M. Fujii, M. E. Zolan, and T. Kamada, 1998 Mutations in clamp-cell formation, the *A*-regulated sexual morphogenesis in homobasidiomycetes, pp. 143-146 in *Proceedings of the Fourth Meeting on the Genetics and Cellular Biology of Basidiomycetes*, edited by L. J. L. D. Van Griensven and J. Visser. Mushroom Experimental Station, Horst, The Netherlands.

Polak, E., 1999 *Asexual Sporulation in the Basidiomycete Coprinus cinereus*. Ph.D. thesis, ETH Zurich, Zurich, Switzerland.

Polak, E., R. Hermann, U. Kües, and M. Aebi, 1997 Asexual sporulation in *Coprinus cinereus*: structure and development of oidiophores and oidia in an *Amut Bmut* homokaryon. Fungal Genet. Biol. **22:**112-126.

Polak E., M. Aebi and U. Kües, 2001 Morphological variations in oidium formation in the basidiomycete *Coprinus cinereus*. Mycol. Res. **105:** 603-610.

Pukkila, P. J., 1993 Methods of genetic manipulation in *Coprinus cinereus*, pp. 249-264 in *Genetics and Breeding of Edible Mushrooms*, edited by S. T. Chang, J. A. Buswell and P. G. Miles. Gordon and Breach Science Publishers, Y-Parc, Switzerland.

Pukkila, P. J., 1994 Meiosis in mycelial fungi, pp. 267-282 in *The Mycota,* Vol. I., edited by J. G. H. Wessels and F. Meinhardt. Springer, New York.

Pukkila, P. J., 1996 Production and analysis of meiotic mutants in *Coprinus cinereus*, pp. 363-369 in *Fungal Genetics:Principles and Practice*, edited by C. J. Bos. Marcel Dekker, New York.

Redhead, S. A., R. Vilgalys, J. M. Moncalvo, J. Johnson, and J. S. Hopple, 2001 *Coprinus* Pers. and the disposition of *Coprinus species* sensu lato. Taxon **50:** 203-241.

Swamy, S., I. Uno, and T. Ishikawa, 1984 Morphogenetic effects of mutations at the *A* and *B* incompatibility factors of *Coprinus cinereus*. J. Gen. Microbiol. **130:** 3219-3224.

Takemaru, T., and T. Kamada, 1969. The induction of morphologenetic variations in *Coprinus* basidiocarps by UV irradiation. Rep. Tottori Mycol. Inst. **7:** 71-77.

Takemaru, T., and T. Kamada, 1970 Genetic analysis of UV-induced, developmental variations in *Coprinus* basidiocarps: the elongationless and expansionless. Rep. Tottori Mycol. Inst. **8:** 11-16.

Takemaru, T., and T. Kamada, 1972 Basidiocarp development in *Coprinus macrorhizus* I: Induction of developmental variations. Bot. Mag. Tokyo **85:** 51-57.

Tsusue, Y. M., 1969 Experiment control of fruit-body formation in *Coprinus macrorhizus* Dev. Growth Differ. **11:** 164-178.

Tymon, A. M., U. Kües, W. V. J. Richardson, and L. A. Casselton, 1992 A fungal mating type protein that regulates sexual and asexual development contains a POU-related domain. EMBO J. **11:** 1805-1813.

Uno, I., and T. Ishikawa, 1971 Chemical and genetical control of induction of monokaryotic fruiting bodies in *Coprinus macrorhizus*. Mol. Gen. Genet. **113:** 228-239.

Walser, P. J., 1997 *Environmental Regulation of Asexual Sporulation and Fruit Body Formation in the Basidiomycete Coprinus cinereus*. Master thesis, ETH Zurich, Zurich, Switzerland.

Walser, P. J., M. Hollenstein, M. J. Klaus, and U. Kües, 2001 Genetic analysis of basidiomycete fungi, pp. 59-90 in *Molecular and Cell Biology of Filamentous Fungi: A Practical Approach*, edited by N. J. Talbot. Practical Approach Series, IRL Press, Oxford.

Walser P. J., R. Velagapudi, M. Aebi, and U. Kües, 2003 Extracellular matrix proteins in mushroom development. Recent Res. Devel. Microbiol. **7:** 381-415.

Wösten, H. A. B., and J. G. H. Wessels, 2005 The emergence of fruiting bodies in basidiomycetes in *The Mycota*, Vol. I, Ed.2., edited by U. Kües and R. Fischer. Springer, Heidelberg, Germany, in press.

Yuki, K., M. Akiyama, M. Muraguchi, and T. Kamada, 2003 The *dst1* gene responsible for a photo morphogenetic mutation in *Coprinus cinereus* encodes a protein with high similarity to WC-1. Fungal Genet Newsl. **50:** Suppl.147. (http://www.fgsc.net/asil2003/asil2003abs.htm).

CHAPTER 3

Construction of co-isogenic strains of *Coprinopsis cinerea* homokaryon AmutBmut with different wildtype mating type specificities

Srivilai, P., Muraguchi, H., and Kües, U.

This manuscript has been prepared for submission to a microbiology journal. All experimental work of this chapter has been preformed by me. Dr. Hajime Muraguchi assisted in advice on mapping by using data from the linkage groups in the published map of the basidiomycete *Coprinopsis cinerea* (established on the basis of random amplified polymorphic DNAs and restriction fragment length polymorphisms; Fungal Genet. Biol., 240:93-102), and helped in selection of suitable primers, respectively molecular gene markers.

3.1. Abstract

Fruiting body development is a process that normally occurs on dikaryons of the basidiomycete *Coprinopsis cinerea*. Homokaryon AmutBmut with specific mutations in the two mating type loci *A* and *B* fruits without mating to another strain. Thus, by mutagenesis, the self-compatible strain allows easy access to dominant as well as recessive genes acting in fruiting. However, so far it was difficult to follow mutated genes in genetic crosses by lack of suitable co-isogenic monokaryons. In this paper, we present such strains with compatible mating type specificities. In course of the co-isogenisation process, we observed an approximately tenfold higher recombination rate between genes on chromosome X carrying the *B* mating type locus and between genes on chromosome I carrying the *A* mating type locus.

3.2. Introduction

Coprinopsis cinerea (formerly *Coprinus cinereus*) is an excellent model fungus to study developmental processes in the basidiomycetes (Kües 2000). *C. cinerea* is accessible to Mendelian genetics (Walser et al. 2001) and DNA transformation (Binniger et al. 1986; Granado et al. 1997) and the genomic sequence has been released to the public by the Broad Institute (see http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/). The fungus performs its complete life cycle within two weeks, also under laboratory conditions. Since classical genetics is principally possible to carry out with the fungus, a genetic map with over 100 genes is available for the species. The map has been established from crosses of various strains coming mostly from European locations (North 1990, Casselton 1995). Another map has independently been created from Japanese strains that differ in some of the chromosome allocations, emphasizing that the genetic backgrounds have a critical role in mapping (Takemaru 1982; North 1990; Casselton 1995). Different backgrounds are however essential for chromosome mapping by molecular means such as karyotyping (Zolan et al. 1994; Arima and Moringa 1995; Zolan 1995; Arima et al. 1996) and RFLP (restriction fragment length polymorphism) and RAPD (random amplified polymorphic DNA) mapping (Ito et al. 1998; Muraguchi et al. 2003). On the contrary, genetics is often hampered by the different genetic backgrounds, for example by loss of parts of progenies due to chromosomal translocations and/or genetic incompatibilities (Moore 1981; Liu et al. 1999; Casselton and Riquelme 2004) and by the frequent presence of (mostly recessive) mutations in the natural *C. cinerea* population (Moore 1981; Kües 2002). In particular, natural occurring genetic variations are known in steps of sexual development such as reciprocal transfer of nuclei between mating monokaryons (May and Taylor 1988), fruiting body formation (Uno and Ishikawa 1971; Moore 1981; Kües et al. 1998; Muraguchi et al. 1999) and basidiospore production and colorization (Pukkila 1993; Kües et al. 2002b). Whilst the natural genetic variability of *C. cinerea* strains certainly presents special challenges (Kües et al. 1998, 2002a, 2002b; Polak et al. 2001), for a clear-cut mutant analysis in Mendelian genetics co-isogenic monokaryotic strains are required that preferentially only distinguish in the mating-type specificities of their haploid nuclei (Raper and Hoffman 1974; Wessels 1999). So far, few attempts have been performed to obtain co-isogenic monokaryons from the model basidiomycete *C. cinerea* (Zolan et al. 1992, 1993; Pukkila 1993).

In the life cycle of *C. cinerea*, monokaryons of different mating type specificities produce upon fusion a dikaryon with two distinct haploid nuclei per hyphal segment (one from each monokaryotic parent) and clamp cells at the hyphal septa (Casselton and Olesnicky 1998; Kües et al. 2002a). Mating type specificity and formation and maintenance of the dikaryon are controlled by the two mating type loci *A* and *B* that encode two classes of homeodomain transcription factors (for

details see reviews by Casselton and Olesnicky 1998 and Hiscock and Kües 1999) and pheromones and pheromone receptors, respectively (Brown and Casselton 2001; Kothe 2001). On the dikaryon, *A* mating type genes of different specificity together regulate clamp cell formation, nuclear pairing and synchronized nuclear division. Different *B* mating type genes control clamp cell fusion (Casselton and Olesnicky 1998; Kües et al. 2002a; Badalyan et al. 2004). Furthermore, the different *A* and *B* mating type genes act in fruiting body initiation and in maturation at the point of induction of karyogamy (Tymon et al. 1992; Kües et al. 1998, 2002b). Therefore, fruiting body development occurs normally on the dikaryotic mycelium (Kües 2000; Kamada 2002).

Strain AmutBmut (*A43mut, B43mut*, *pab1-1*) is a self-compatible homokaryon whose nuclei carry defects in both mating type loci (Swamy et al. 1984). In consequence, it forms fruiting bodies without the need to mate to another strain (Boulianne et al. 2000; Kües et al. 2004). Homokaryon AmutBmut has been proven to be very valuable for producing both dominant and recessive mutants in fruiting body and basidiospore development by traditional UV and modern REMI mutagenesis (Chiu and Moore 1990; Pukkila 1994; Granado et al. 1997; Cummings et al. 1999; Liu 2001; Lu et al. 2003). UV-mutants can directly be submitted to transformation with a suitable cosmid library (Bottoli et al. 1999) in order to search for genes complementing their defects (Liu et al. 2006; Clergeot et al., unpublished). However, mutants may contain more than one defect, complicating the analysis (Liu et al. 2006). REMI (restriction enzyme-mediated integration) mutants, created by DNA transformation in presence of restriction enzymes, may carry more than one DNA insert in the genome (Liu et al. 1999) hindering easy access on inactivated genes. Therefore, outcrossing of mutations of interest is advisable.

The self-compatible strain AmutBmut can be mated with wildtype monokaryons carrying other mating type specificities to give normal dikaryons. In contrast, in crosses with strains of related mating type (e.g. two different AmutBmut mutants), selection is not strong enough to keep together the haploid nuclei of the two mating partners within the cells of growing mycelium. As a consequence of this, complementation might be observed in the mating zone between mutants that have defects in early steps of fruiting body development (hyphal knot formation), but not between mutants with defects in later steps of fruiting body development (Liu et al. 1999). The latter requires that the different nuclei are passed on from the cells of the hyphal knots generated at the mycelial fusion zones to the tissues of the developing fruiting body and, finally, to the specialized basidia in which karyogamy and meiosis occur (Walser et al. 2003; Kües et al. 2004). Finding after meiosis only one type of nuclei within the basidiospores of a given fruiting body suggests this not to happen between self-compatible mutants (Liu et al. 1999). A way out of the problem to access mutations in later fruiting stages is to construct monokaryons co-isogenic to homokaryon AmutBmut with different wildtype mating type specificities.

3.3. Material and methods

3.3.1. *C. cinereus* strains and cultural conditions

The *para*-aminobenzoic acid (PABA)-auxtrophic homokaryons AmutBmut (*A43mut*, *B43mut*, *pab1-1*) and 326 (*A43mut*, *B43mut*, *pab1-1*) and monokaryons 218 (*A3*, *B1*, *trp-1.1,1.6*, *bad*), JV6 (*A42*, *B42*), PG78 (*A6*, *B42*, *pab1*, *trp1.1,1.6*), MK45 (*A43*, *B1*, *ade8*) and KF_3#2 (*A91*, *B91*) were used in this study (Kertesz-Chaloupková et al. 1999; Kües et al. 2001; Muraguchi et al. 2003). For crosses and fruiting tests, strains were grown at 37°C in the dark on solid YMG/T complete medium. For induction of fruiting body formation, fully grown cultures were transferred into 25°C and a 12h dark/12h light rhythm. Basidiospores were harvested and plated on YMG/T or minimal medium MM as required (Granado et al. 1997; Walser et al. 2001). Small pieces of germinated clones (ca. 1x2 mm^2) were transferred to fresh YMG/T plates for growth for 36 h at 37°C to analyze colony features. Clamp cell production, clamp cell fusion and dikaryon formation served to follow up inheritance of mating type genes. *A43mut* homokaryotic strains were recognized by production of clamp cells at hyphal septa, clones with wildtype *A* mating type genes by lack of clamp cells. *B* alleles in clones with wildtype *A* mating type genes and in clones with the *A43mut* allele were identified by crosses with suitable monokaryons, the *B43mut* allele in addition by observing clamp cell fusion in *A43mut* clones (Swamy et al. 1984; Kües 2000). Recombinants between *A* mating type genes and *pab1* were identified by testing clones for growth on MM and analyzing hyphal septa by microscopy.

The following strains obtained in this study were stored for further experiments: *A42*, *B42* clone PS001 from generation F5 of cross JV6 x AmutBmut (used to generate F6) and *A42*, *B42* clones PS001-1 to PS001-7, *A42, B43mut* clones PS001-8 to PS001-11 and the *A42*, *B42*, *pab1-1* clone GAU1 from generation F6; *A3*, *B1* clone PS002 from generation F5 of cross 218 x AmutBmut (used to generate F6) and *A3*, *B1* clones PS002-1 to PS002-5 and PS002-12 to PS002-14, *A3*, *B43mut* clones PS002-6 to PS002-11 and PS002-15 to PS002-24, the *A3*, *B43mut*, *pab1-1* clone GAU2 and the *A43mut*, *B43mut*, *pab1*$^+$ clone OU2 from generation F6; UFO1 (*A43mut*, *B43mut*, *pab1-1*, *eln*) from cross 218 x AmutBmut; PS003-1 (*A3*, *B42*) and PS003-2 (*A42*, *B1*) from cross PS001-1 x PS002-1; PS004-1 (*A3*, *B42*) and PS004-2 (*A42*, *B1*) from cross PS001-2 x PS002-1; PS005-1 (*A3*, *B42*) and PS005-2 (*A42*, *B1*) from cross PS001-2 x PS002-12.

3.3.2. Calculation of genetic identity

The formula of Leslie (1981): $C(n) = 2c\,(1/2)^{n} = c\,(1/2)^{n-1}$ predicts the average number of chromosome tips remaining allogenic in repeated backcrosses with random chromosome distribution. C (n) is the average number of chromosome tips remaining allogenic, c the haploid chromosome number (13 in *C. cinerea*; Pukkila and Lu 1985) and n the number of backcrosses performed.

3.3.3. DNA methods

Genomic DNA was extracted as described by Zolan and Pukkila (1986). RAPD analysis with a selection of different 10-mers was performed with strains JV6, 218 and AmutBmut, and, for control, with strains 326 and KF_3#2. PCR conditions were as described before (Muraguchi et al. 2003). Names of 10-mers used are given in the results section. For their origin and sequence, see Ito et al. (1998) and Muraguchi et al. (2003). *coh5-coh4* spacer regions were amplified with primers *coh5*f 5´CAAAGTTCTCTCTACCC3´ and *coh4*r 5´CGTTAATATAATCACCCGA3´ (Velagapudi 2006). *lcc15* sequences were amplified with primers Abgpd_lcc15 5´CTCCCATCTACACA-CAACAAGCTTATCGCCATGCATCCCCACCTTCGA3´ and lcc1term_lcc15 5´ACTGGCCCT-CTGGTCAACTATAATATTATCTAGAAGGGAATGTTGGTTG3´ (Kilaru *et al.* 2006).

3.4. Results

3.4.1. Construction of strains from *A42, B42* monokaryon JV6 that are co-isogenic to homokaryon AmutBmut

Monokaryon JV6 (*A42*, *B42*) with a sturdy dense aerial mycelium was mated to the fluffy growing homokaryon AmutBmut (*A43mut*, *B43mut*, *pab1-1*). After fruiting body formation, spores were collected and plated on MM to counter-select *pab1-1* clones and, with it, in most cases the closely linked *A43mut* mating type allele (North 1990). Germinated clones were transferred to YMG/T medium. The *B* mating type specificities within the obtained clamp-less *A42* progeny were determined by crosses to the *A6*, *B42* monokaryon PG78 and found to be distributed in a 1:1 pattern. Irrespectively of the *B* mating type specificity, *A42* clones of the F1 progeny differed very much in colony morphologies (growth diameters, mycelial densities, appearances of aerial mycelia; Table 1; Fig. 1, left), reflecting the heterogeneous genetic backgrounds of the Javanese strain JV6 (also known as Java6; May and Matzke 1995) and the Japanese homokaryon AmutBmut (Swamy et al. 1984).

A random *A42*, *B42* F1 clone with a colony appearance and growth behaviour similar to homokaryon AmutBmut (growth 7.5 mm per day on YMG/T at 37°C) was selected and backcrossed to the parental strain AmutBmut. *A42*, *B42* and *A42*, *B43mut* clones were identified in analogy as described for the F1 generation. The *B* alleles distributed in a 1:1 pattern within the *A42* F2 progeny as in all further generations obtained from backcrosses of *A42*, *B42* clones to homokaryon AmutBmut (Table 1).

Already in the F2 generation, morphologies of most colonies were very similar to each other and resembled that of homokaryon AmutBmut. However, two major types of growth speed emerged. After incubation on YMG/T medium for 36 h at 37°C, colony diameters of faster growing clones were in the range of 1.9-2.3 cm (values from the F5 generation: 2.12 ± 0.17 cm, n = 32 and from the F6 generation: 2.16 ± 0.12 cm, n=47) and colony diameters of slower growing strains in the range of 1.5-1.8 cm (values from the F5 generation: 1.57 ± 0.10 cm, n = 18 and from the F6 generation: 1.65 ± 0.10 cm, n =13). The *B42* mating type correlated in most instances with faster growth, whereas clones carrying the *B43mut* genotype usually showed retarded growth (Table 1, Fig. 1). With higher number of filial generations (F5, F6), the differences in growth speed between groups of clones with the *B42* mating type and groups of clones with the *B43mut* mating type were less pronounced but did not fully disappear. The mycelium of colonies of the F5 and F6 generations were studied under the microscope. Most hyphae did not show obvious morphological differences between the *B42* and *B43mut* clones but in *B43mut* colonies unusual hyphae with zigzag growth were regularly observed (Fig. 2). In contrast, in colonies with the wildtype *B42* mating type genes, at the most, very few hyphae may grow somewhat wavy (not shown).

Table 1 Colony growth phenotypes* in filial generations from cross JV6 x AmutBmut

Mating type	***A42, B42***			***A42, B43mut***		
Clones	**Number of small colonies**	**Number of large colonies**	**Total clones**	**Number of small colonies**	**Number of large colonies**	**Total clones**
F1	14	14	28	21	8	29
F2	2	13	15	11	3	14
F3	10	19	29	21	8	29
F4	4	21	25	17	9	26
F5	3	35	38	20	21	41
F6	3	40	43	18	24	42

*Colony diameters were measured after 36 h of growth at 37°C on YMG/T medium and arbitrarily defined as small if 1.8 cm and as large if 1.9 cm.

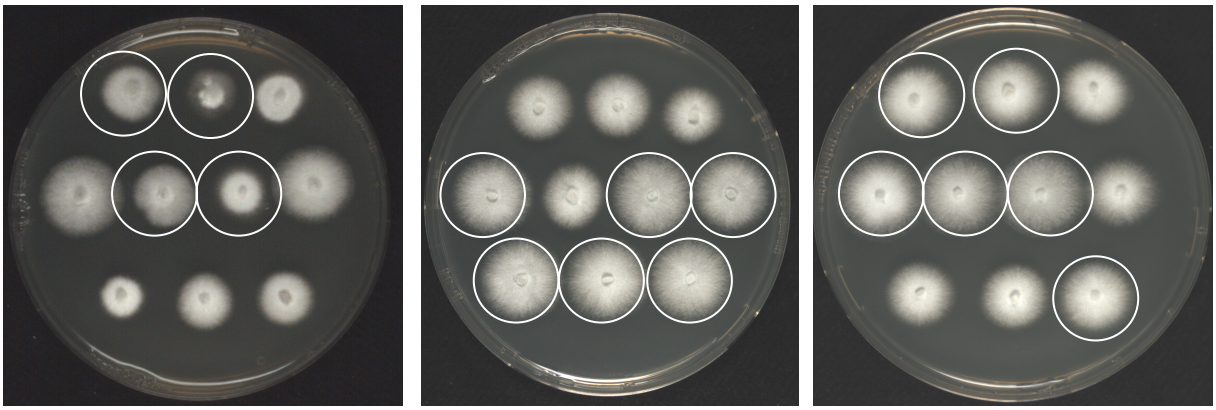


Figure 1. Randomly selected *A42* clones of the F1, F5 and F6 progenies from cross JV6 x AmutBmut (from left to right). Clones that carry the *B42* mating type genes are encircled.

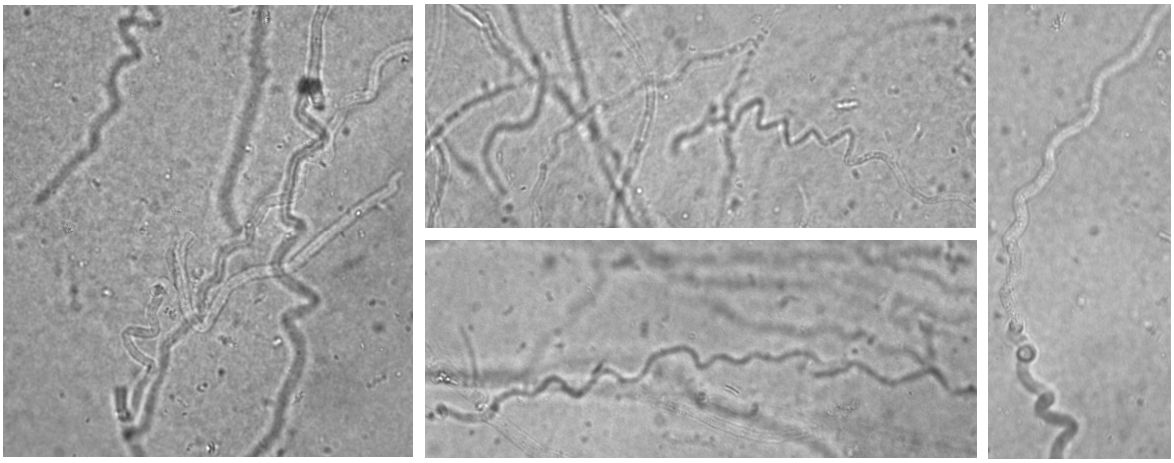


Figure 2. Hyphae with zigzag growth morphology found regularly in *A42*, *B43mut* clones of the F6 progeny from cross JV6 x AmutBmut.

3.4.2. Construction of strains from *A3, B1* monokaryon 218 that are co-isogenic to homokaryon AmutBmut

Monokaryon 218 (*A3*, *B1*, *trp1.1,1.6*, *bad*) was mated to the *A43mut*, *B43mut*, *pab1-1* homokaryon AmutBmut and fruiting bodies induced. Resulting spores were plated on MM, thereby counter-selecting the 218 *trp1.1,1.6* defect on linkage group IV and the AmutBmut *pab1-1* defect linked to the *A* mating type locus on chromosome I (Muraguchi et al. 2003). Germinated F1 clones were transferred to YMG/T plates. They were crossed with the *A43, B1* monokaryon MK45 to distinguish *B1* and *B43mut* strains by dikaryon formation, respectively. Five further rounds of backcrosses of selected *A3*, *B1* clones to homokaryon AmutBmut were performed and, in each case, *A3* progeny was pre-selected by plating spores on MM. Within all filial generations, *B1* and *B43mut*

distributed in a 1:1 pattern (data not shown). Within the F1 progeny, the morphology of individual clones was somewhat variable. Starting with F2, the majority of clones resembled each other in colony appearance (not shown). Nevertheless, colony growth diameters varied. 80-90% of the clones with the *B1* mating type in generations F2 to F6 formed after 36 h growth on YMG/T medium at 37°C colonies with diameters in the range of 1.8-2.2 cm (mean values calculated for the F5 generation: 1.89 ± 0.10 cm, n = 32 and for the F6 generation: 1.86 ± 0.13 cm, n = 40). 45-60% of the clones carrying the *B43mut* allele in generations F2 to F6 gave smaller colony diameters in the range of 1.3-1.7 cm (mean values calculated for the F5 generation: 1.44 ± 0.17 cm, n = 28 and for the F6 generation: 1.49 ± 0.07 cm, n = 19) after 36 h growth on YMG/T medium at 37°C. Analyses of *A3* clones of the F5 and F6 generation of cross 218 x AmutBmut under the microscope revealed that zigzag hyphae occurred in *B43mut* and in *B1* strains in similar frequencies as in the *A42*, *B43mut* colonies from the F5 and F6 generations of the JV6 x AmutBmut cross (not shown).

Furthermore in the F6 generation, we found one recombinant strain with the genotype *A43mut*, *B43mut*, *pab1*$^{+}$ (OU2) amongst 100 *pab1*$^{+}$ clones analyzed. Due to the activated *A* mating pathway, strain OU2 was fast growing (colony Ø of 2.2 cm after 36 h growth at 37°C), had on YMG/T-plates a slightly more fluffy colony morphology than the clones with the wildtype *A* mating type genes and produced fruiting bodies with basidiospores.

3.4.3. Recombination between the *A* mating type locus and the *pab1* gene

Our goal in this study was to isolate both, prototrophic and *pab1*-auxotrophic co-isogenic strains with wildtype *A* mating type genes in order to be as versatile as possible in future analyses of *pab1*-auxotrophic UV-mutants and of prototrophic REMI mutants of homokaryon AmutBmut being transformed with the *C. cinerea pab1*$^{+}$ gene (Granado et al. 1997). Recombination events between the *A* mating type locus and the closely linked gene *pab1* were examined in the F6 generations by plating basidiospores on YMG/T medium and transferring germinated clones onto MM. Clones with a *pab1* defect were able to grow sparsely under these conditions due to the small amount of PABA transferred with the YMG/T inoculums. Hyphae of these clones were analyzed for lack of clamp cell production. In the F6 generation from cross JV6 x AmutBmut, one recombinant strain (GAU1 with a colony diameter of 1.9 cm after 36 h growth at 37°C; genotype *A42*, *B42*, *pab1-1*) was found in a total of 213 *pab1*-defective analyzed clones. Likewise, in the F6 generation from cross 218 x AmutBmut, one strain (GAU2 with a colony diameter of 1.6 cm after 36 h growth at 37°C; genotype *A3*, *B43mut*, *pab1-1*) of a total of 269 *pab1*-defective analyzed clones was recombinant. The calculated recombination frequencies (0.47 and 0.37, respectively) were comparable to the published map distance of 0.5 map units between the *pab1* gene and the *A* mating type locus (Day 1960).

3.4.4. The level of genetic identity

In our initial cross of homokaryon AmutBmut with monokaryon 218, we positively selected the *trp1*$^{+}$ marker from homokaryon AmutBmut on linkage group IV. The *A* and the *B* mating type genes on linkage groups I and X (Muraguchi et al. 2003) positively selected for in all crosses were from strain 218. For the 10 remaining randomly selected chromosomes, by the formula of Leslie (1981), we can expect to have in the F6 generation in average only 0.3125 chromosome tips still remaining from strain 218 (i.e. about 1.5% of genes on the 10 chromosomes are expected to be allogenic). In the case of the cross JV6 x AmutBmut, chromosome III with the *trp1* locus has in addition to be considered in the calculation of the average number of allogenic tips from the randomly selected chromosomes. Accordingly, in the F6 generation in average only 0.344 chromosome tips and 1.7% of genes on the 11 non-mating type chromosomes are expected to come from strain JV6. Since for generating subsequent filial generations clones were selected according to colony morphology most similar to homokaryon AmutBmut, it is possible that even more genetic identity is given in the group of apparently randomly selected chromosomes.

3.4.5. Inheritance of the fruiting ability of homokaryon AmutBmut

Only small parts of *A43mut, B43mut* F1 progenies from crosses of homokaryon AmutBmut with unrelated monokaryons (monokaryon PG78 and others) tend to give rise to mature fruiting bodies (Liu et al. 1999; Liu 2001). In this study, we confirmed this former observation also for monokaryons JV6 and 218 (Table 2). Only 43% of *A43mut, B43mut* F1 clones from cross JV6 x AmutBmut and 65% from cross 218 x AmutBmut initiated fruiting and only 17%, respectively 51% completed fruiting. In accordance with a defect in spore formation gene (*bad*) in monokaryon 218 (Pukkila 1993; Kües et al. 2002), half of the clones with fully developed fruiting bodies from the cross 218 x AmutBmut produced no basidiospores. Furthermore in the F1 progeny of cross 218 x AmutBmut, we isolated one *A43mut*, *B43mut* clone (UFO1) that in addition to the *bad* gene from monokaryon 218-carried a spontaneous mutation *eln* leading to ufo-shaped primordia and short mushrooms due to lack of stipe elongation (Srivilai et al. 2005).

In the higher filial generations of crosses JV6 x AmutBmut and 218 x AmutBmut, along with a more unique colony morphology, the frequency of *A43mut, B43mut* clones forming mature fruiting bodies raised dramatically, up to values of around 90% (Table 2), indicating a high degree of co-isogenisation in the randomly selected chromosomes. No white cap mushrooms without spores were observed in later progenies of cross 218 x AmutBmut, showing that the *bad* gene from monokaryon 218 was eliminated during backcrossing *A3*, *B1* clones to homokaryon AmutBmut.

Table 2 Fruiting behaviour of *A43mut B43mut* progeny from crosses of homokaryon AmutBmut with monokaryons JV6 and 218 and monokaryons derived from filial generations F5 and F6 of cross JV6 x AmutBmut and cross 218 x AmutBmut

Cross	Total progeny tested	AmutBmut mycelial appearance	No fruiting initiation	Primordia formation	Fruiting bodies	
					White	Black
JV6 x AmutBmut	54	11 (20%)	31 (57%)	14 (26%)	-	9 (17%)
PS001 (F5) x AmutBmut	68	61 (90%)	15 (22%)	13 (19%)	-	40 (59%)
PS001-1 (F6) x AmutBmut	52	52 (100%)	3 (6%)	4 (8%)	-	45 (87%)
218 x AmutBmut	79	26 (33%)	28 (35%)	11 (14%)	19* (24%)	21 (27%)
PS002 (F5) x AmutBmut	70	64 (91%)	10 (14%)	12 (17%)	-	48 (69%)
PS002-1 (F6) x AmutBmut	56	56 (100%)	3 (5%)	2 (4%)	-	51 (91%)

* Includes strain UFO1

3.4.6. Crosses between *A42*, *B42* and *A3*, *B1* monokaryons of the F6 generations

Compared to the different filial progenies of cross JV6 x AmutBmut, those of cross 218 x AmutBmut were in average always slightly reduced in growth (see above). The *A42*, *B42* clone PS001-1 (colony diameter 2.0 cm after 36 h growth on YMG/T at 37°C) from the F6 generation of cross JV6 x AmutBmut was crossed with the *A3*, *B1* clone PS002-1 (colony diameter 1.8 cm after 36 h growth on YMG/T at 37°C) from the F6 generation of cross 218 x AmutBmut. Progeny of this cross was obtained by plating basidiospores on YMG/T medium. Within the tested progeny, *A3* clones were somewhat underrepresented (37 % compared to 63 % of clones carrying the *A42* mating type; total number of clones analyzed: 73) whilst the *B* mating type alleles were evenly distributed (see Table 3). Growth was best amongst *A42*, *B42* clones, followed by *A3*, *B1* clones (Table 3). Most strains with new combinations of mating type specificities (*A42*, *B1* and *A3*, *B42*, respectively) were reduced in growth (Table 3). It appears that functions linked to the *A* and functions linked to the *B* mating type genes are responsible for differences in growth behavior.

Table 3 Colony growth phenotypes* in the progeny of cross PS001-1 x PS002-1

Genotype	Number of small colonies (mean Ø of colonies in cm)	Number of large colonies (mean Ø of colonies in cm)	Total clones (mean Ø of colonies in cm)
A42, B42	6 (1.52 ± 0.16)	17 (1.99 ± 0.08)	23 (1.87 ± 0.24)
A42, B1	18 (1.59 ± 0.12)	4 (1.93 ± 0.05)	22 (1.65 ± 0.17)
A3, B1	8 (1.55 ± 0.13)	7 (1.91 ± 0.04)	15 (1.72 ± 0.21)
A3, B42	10 (1.49 ± 0.25)	3 (1.90 ± 0.04)	13 (1.58 ± 0.28)
Total clones	42	31	73

*Colony diameters were measured after 36 h of growth at 37°C on YMG/T medium and arbitrarily defined as small if 1.8 cm and as large if 1.9 cm.

3.4.7. Molecular proof of genetic identity by RAPD analysis

A selection of different 10-mers used to create a RAPD map for strains 326 and KF_3#2 (Ito et al. 1998; Muraguchi et al. 2003) were tested for suitability to follow up chromosomes of strains AmutBmut, 218 and JV6 in their genetic progenies.

Homokaryon 326 is related to both strains AmutBmut and 218. It has been created from these strains by repeated backcrossing of progeny to strain 218 (P. J. Pukkila, personal communication). Therefore, subgroups from the RAPD markers assigned to linkage groups of strain 326 (B fragments in the published RAPD map) by Muraguchi et al. (2003) are expected to be found also in monokaryon 218 and/or in homokaryon AmutBmut. In consistency, 10-mers A7, A11, A12, A14, BA3, BG4, BG5, E3, G11, G14, G15, Q1, Q5, R8, S1, S3 and S11 produced indistinguishable banding patterns in PCR with genomic DNAs from the three strains. Nearly identical patterns were obtained for the three strains with primers A3, A4, E2, G17 and S19. Primers A5, A20, G10, Q6, Q17, R7, S9, S13 and S14 gave identical patterns for strains 218 and 326 that partially overlapped with those from homokaryon AmutBmut. Primer G18 gave an identical pattern for strains 218 and 326 that differed from that of homokaryon AmutBmut. Patterns for A10, E19, G5, G19, Q15 and S12 were identical for strains AmutBmut and 326 and partially with those of monokaryon 218.

Patterns for primers A9, BA4 and E4 were identical and the pattern for primer G3 similar for strains AmutBmut and 326 and different from those of monokaryon 218. Partially identical patterns for the three strains were obtained with primers A2, E5, E7, E18, Q3, Q18, R4, R8, G4, G14, BA3, BA5 and R9. Nevertheless, in a few cases, RAPD bands discriminated locations from seven different chromosomes between strains 218 and AmutBmut (chromosomes I, II, IV, VI, VIII, IX, and X) and eight further locations not assigned to a specific chromosome (Table 4). Marker distribution within the tested F6 progeny of cross 218 x AmutBmut (11 different fully tested clones) was identical to homokaryon AmutBmut with exception of the two RAPD bands G19-1700+ and A10-1400+ that occurred only in parts of the progeny (Table 4). Variation in inheritances of these two bands was also seen in 13 further tested clones (PS002-12 to PS002-24). Furthermore, clone PS002-11 had a new 1.3 kb band instead of the not further mapped band A5-1500+ found in both parental strains 218 and AmutBmut (Table 4).

Using the same set of primers as above, monokaryon JV6 in several instances gave a (completely) different banding pattern in PCR amplification than strains AmutBmut, 326 and 218. Only primers A14, BA3, BG4, BG5, Q1 and S3 produced with strain JV6 patterns that were identical and with primers A2, A3 and BA4 patterns that were similar to those from homokaryons AmutBmut and 326. Partially conserved patterns between the three strains were obtained with primers A4, A5, A7, A10, A20, E2, E4, E5, E19, Q6, Q15, Q18, R4, S11, S12, S13 and S14. Primers Q5, R5 and S9 gave patterns with JV6 DNA similar to monokaryon KF_3#2. In conclusion, RAPD markers for all chromosomes but I were found that can be followed in the progenies of cross JV6 x AmutBmut (Table 4). In the F6 progeny, in all instances the RAPD pattern was as in homokaryon AmutBmut in but for RAPD bands G19-1700+, A10-1400+ and E4-1400+ (Table 4; Fig.3). The results suggest an overall high genetic homogeneity.

Table 4 Marker analysis

Chromosome[1] Marker	**KF_3 #2**	**326**	**JV6**	**218**	**AmutBmut**	**F6 Progeny JV6 x AmutBmut[2]**	**F6 Progeny 218 x AmutBmut[2]**
I							
A3	−	−	−	+	−	−	+
A42	−	−	+	−	−	+	−
pab1-1	−	+	−	−	+	GAU1 +, all others −	GAU2 +, all others −
BA4-3100B=	−	+	+	−	+	−	−
1.5 kb lcc15	+	−	+	−	−	PS001-5, -10 −, all others +	−
3.0 kb lcc15	−	+	−	+	+	PS001-5, -10 +, all others −	+

Table 4 (continued)

II							
G5-1700B+	−	+	−	+	+	+	+
A5-1700B+	−	+	−	+	−	−	−
G14-850B=[3]	−	+	−	+	+	+	+
E7-1000A+	+	−	+	−	+	+	+
III							
S19-900B+[3]	−	+	−	+	+	+	+
G2-1000B-	−	+	−	+	+	+	+
S12-1800B+[3]	−	+	−	+	+	+	+
G10-1100B+[3]	−	+	−	+	+	+	+
IV							
trp1.1,1.6	−	−	−	+	−	−	−
A9-1800B=	−	+	−	−	+	+	+
R4-2300B=	−	+	−	+	+	+	+
V							
G19-3000B=	−	+	−	+	+	+	+
VI							
G10-1800B-[3]	−	+	−	+	−	−	−
A11-400B+	−	+	−	+	+	+	+
VII							
G19-800B=	−	+	−	+	+	+	+
Q17-2500+	−	+	−	+	+	+	+
VIII							
A12-2500B+	−	+	−	+	+	+	+
R9-600B+	−	+	−	−	+	+	+
E19-350B+	−	+	+	−	+	+	+
IX							
A4-500B+	−	+	−	+	+	+	+
A2-1000B+	−	+	+	−	+	+	+
A11-1600B+	−	+	−	+	+	+	+
X							
E4-1000B+	−	+	−	+	+	+	+
E2-1800B+	−	+	+	−	+	+	+
A10-1600B+	−	+	−	+	+	+	+
G19-1700+	−	+	−	−	+	PS001-2, -5, -8, -10 +, all others −	PS002-6, -9, -11 to -14, GAU2 +, all others –
A10-1400+	−	+	−	−	+	PS001-2, -5, -8, -10 +, all others −	PS002-9, -12 to -14, GAU2 +, all others –
E4-1400+	−	+	−	+	+	PS001-2, -5, -7, -8 +, all others −	+
1.75 kb *coh5-coh4* spacer	−	+	−	+	+	PS001-2, -5, -10 +, all others −	+

Table 4 (continued)

X							
1.8 kb *coh5-coh4* spacer	+	−	+	−	−	PS001-2, -5, -10 −, all others +	−
B1	−	−	+	−	−	−	PS002-1 to -5, -12 to -24 +, PS002-6 to -11, GAU2 −
B42	−	−	−	+	−	PS001-1 to -7, GAU1 +, PS001-8 to -11 −	−
B43mut	−	+	−	−	+	PS001-1 to -7, GAU1 −, PS001-8 to -11 +	PS002-1 to -5, -12 to -24 −, PS002-6 to -11, GAU2 +,
XI							
E3-1900B+	−	+	−	+	+	+	+
G17-3000B-	−	+	−	+	+	+	+
XII							
S12-2800A=	+	−	+	−	−	−	−
XIII							
A11-800B+	−	+	−	+	+	+	+
Not further mapped							
G11-2600+[3]	−	+	−	+	+	+	+
A5-1500+	−	+	−	+	+	+	PS002-11 −, all others +
A10-2000+	+	+	−	+	+	+	+
A9-3300-	−	+	−	−	+	+	+
BA4-1700+	+	+	−	−	+	+	+
E7-800+	+	−	+	−	+	+	+
Q17-1050+	−	−	+	−	+	+	+
G10-1900-[3]	−	−	−	−	+	+	+
A2-2200+	−	+	−	+	−	−	−
E19-4000+	−	−	−	+	−	−	−
G17-1200+	−	−	−	+	−	−	−
A10-1450+	+	−	+	−	−	−	−
A2-2250+	−	−	+	−	−	−	−
BA4-1400+	−	−	+	−	−	−	−
R9-800+	−	−	+	−	−	−	−

[1] Chromosome nomenclature as in Muraguchi et al. (2003).
[2] Chromosomal DNA of PS001-1 to PS001-11 and GAU1 from the F6 progeny of cross JV6 x AmutBmut and chromosomal DNA of PS002-1 to PS002-11 and GAU2 from the F6 progeny of cross 218 x AmutBmut were tested with all primers whereas PS002-12 to PS2-24 were only tested with primers A10, E4 and G19.
[3] Clones PS001-1 to PS001-4, PS001-8, PS001-9, GAU1 and clones PS002-1, PS002-2, PS002-7 to PS002-9 and GAU2 were tested.

3.4.8. Molecular markers linked to the *B* mating type locus

Inheritance of RAPD bands G19-1700+, A10-1400+ and E4-1400+ (all newly described in this study) in the F6 progeny of cross JV6 x AmutBmut and of G19-1700+ and A10-1400+ in the F6 progeny of cross 218 x AmutBmut correlated in most instances which suggested linkage between these three RAPD markers (Table 4). Moreover, these differential bands occurred only in about 25-30% of the tested clones with wildtype *B* mating types (Table 4). When we checked 10 different *A42* clones of the F1 progeny of cross JV6 x AmutBmut, respectively 10 different *A3* clones of the F1 progeny of cross 218 x AmutBmut, the markers showed again linkage with each other as well as a loose linkage with the *B43mut* mating type (ca. 30 % recombination rate; not shown).

We know from the released genome sequence of *C. cinerea* strain Okayama 7 (see http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/), that a cluster of seven different hydrophobin genes including *coh1* and *coh2* described before by Ásgeirsdóttir et al. (1997) localizes in a distance of about 120 kb to the *B* mating type locus on contig 1.134 of the strain (Velagapudi 2006). A primer pair specifically amplifying the spacer region between hydrophobin genes *coh5* and *coh4* in PCR resulted with different strains in a fragment length polymorphism. A 1.8 kb fragment was correctly amplified from strain Okayama 7. Fragments of the same size were obtained from strains JV6 and KF_3#2. Strains 218, AmutBmut and 326 each gave rise to a smaller fragment sized 1.75 kb. Inheritance of this 1.75 kb *coh5-coh4* spacer region linked in the tested F1 and F6 generations of cross JV6 x AmutBmut to the RAPD bands G19-1700+, A10-1400+ and E4-1400+ (Table 4), supporting that these are linked to the *B43mut* locus in homokaryon AmutBmut.

Unlike monokaryon JV6, the complete *B42* F6 progeny of cross JV6 x AmutBmut carried A10-1600B+ and E4-1000B+ (Fig. 3). These markers map on chromosome X at distances of 12.8, respectively 25.3 map units to the *B* mating type locus (Muraguchi et al. 2003) and reside in the assembled contig map of chromosome X on the opposite side of the *B* mating type locus than the hydrophobin gene cluster (Muraguchi, unpublished; http://www.akita-pu.ac.jp/bioresource/dbt/cellbiol/muraguchi/Chr1.html). Therefore, recombination must have occurred during our isogenisation process also at that other side of the *B* mating type locus. Recombination must have also occurred at a similar place in chromosome X of strain 218, since unlike this strain, all *B1* F6 progeny of cross 218 x AmutBmut carried E2-1800B+ (Table 4) that localizes in between markers A10-1600B+ and E4-1000B+, 20.3 map units apart from the *B* mating type locus (Muraguchi et al. 2003).

3.4.9. Molecular markers linked to the *A* mating type locus

For chromosome I carrying the *A* mating type locus, we unfortunately did not find suitable RAPD markers to follow up recombination events. The only differential RAPD marker BA-3100B= (Table 4) was shown before to be tightly linked to the *A43mut* mating type genes (Muraguchi et al. 2003). Fortuitously, laccase gene *lcc15* exhibits a fragment length polymorphism (M. Navarro-González and S. Kilaru, personal communication) and we determined that this gene links to the *A* locus at a distance of 20 map units (Table 4). *lcc15* is found on contig 1.28 of the Okayama 7 genome as a gene with an internal deletion (Kilaru et al. 2006). From the ongoing assembling of contigs to individual chromosomes, *lcc15* should localize on chromosome I at distances of about 900 kb to *pab1* and 940 kb to the *A* mating type locus (Muraguchi, unpublished; http://www.akita-pu.ac.jp/bioresource/dbt/cellbiol/muraguchi/Chr1.html).

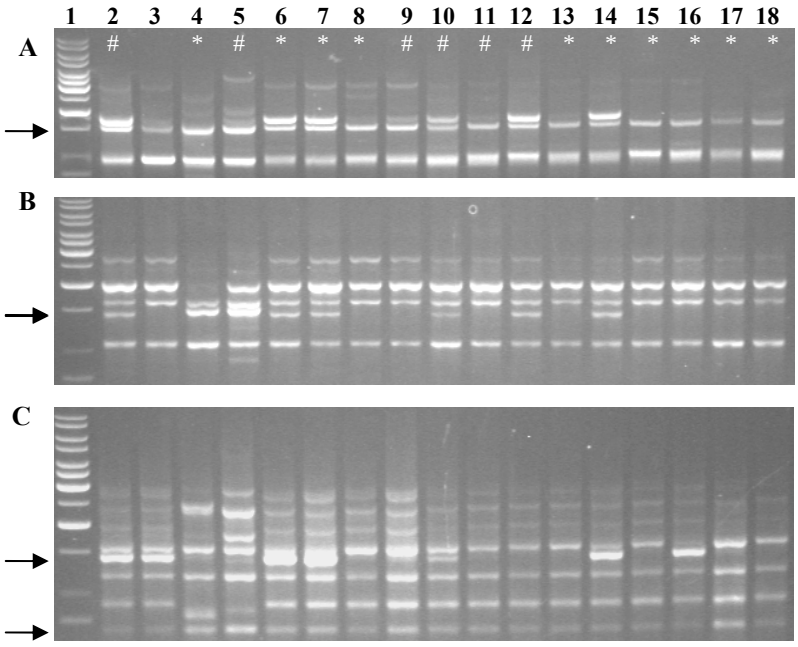


Figure 3. RAPD bands generated from chromosomal *C. cinerea* DNA by primers G19 (**A**), A10 (**B**) and E4 (**C**). The arrows indicate the newly identified *B*-linked bands G19-1700+, A10-1400+ and E4-1400+ and the formerly mapped *B*-linked bands A10-1600B+ and E4-1000B+, respectively. Lane 1: molecular size marker (1 kb ladder); lane 2 to lane 18: homokaryon AmutBmut, monokaryon 218, monokaryon JV6, monokaryon KF_3 # 2, homokaryon 326, PS001-2, PS001-3, PS001-4, PS001-8, PS001-9, PS001-10, PS001-11, PS001-5, PS001-6, PS001-7, GAU1, PS001-1. *indicates *B42* strains, # *B43mut* strains.

3.5. Discussion

Co-isogenic strains differing insofar possible only in their mating types have been produced in the basidiomycete model fungus *Schizophyllum commune* decades ago in the Raper lab (Raper 1966; Raper and Hoffman 1974). These strains are wide-spread used in research because they offer clear-cut results in genetic analysis (Wessels 1999; Raper and Hoffmann 1974). Earlier genetic work with *S. commune* strains of diverse backgrounds was hampered by loss of parts of progenies due to irregularities in meiosis (Raper and Oettinger 1962; Wessels 1999) and by wide-ranging natural variations in developmental processes such as in fruiting (Raper and Krongelb 1958). Similar problems were also faced in *C. cinerea* (Moore 1981; Liu et al. 1999; Liu 2001; Kües et al. 1998). With the newly created monokaryons co-isogenic to *C. cinerea* homokaryon AmutBmut and of compatible mating types, genetic analyses of fruiting mutants of AmutBmut background promise to become much more straightforward. As a first successful example, mutations at three different stages in fruiting body development in UV-mutant 6-031 (*skn*, *mat* and *bad*) have been defined and separated from each other (Liu et al. 2006).

Our phenotypic analysis (growth and fruiting behaviour) and our molecular marker analysis of the F6 progenies of crosses JV6 x AmutBmut and 218 x AmutBmut suggest that we have reached high levels of isogenisation. Nevertheless, there appears still to be some variation amongst genes linked to the mating type loci. For our primary target, genetic analysis of fruiting mutants originating from homokaryon AmutBmut, these variations seem to be of minor importance. With monokaryons from the F6 generations, we achieved for *A43mut*, *B43mut* clones fruiting abilities of 90%, values high enough for mutant analysis (Liu et al. 2006). Some recombination between the genes left variable on the mating types determining chromosomes may account for the moderate, tolerable shortfall in fruiting. Furthermore, homokaryon UFO1 with a spontaneous *eln* defect in stipe elongation and the new RAPD band A5-1300+ in clone PS002-11 of the F6 progeny of cross 218 x AmutBmut show that unpredictable events of mutation and/or recombination happen to some extend in *C. cinerea* in course of meiosis. It is thus possible, that complete fruiting in a progeny will never be obtained.

25-30% of clones with wildtype *B* mating type genes within the F1 and the F6 generations carried some or all of the molecular markers 1.75 kb *coh5-coh4* spacer region, G19-1700+, A10-1400+ and E4-1400+ from homokaryon AmutBmut. By the comparably short distance of the hydrophobin genes to the *B* mating type locus (120 kb), the available data suggest that there is a recombination hotspot close to the *B* mating type genes on chromosome X. Recombination in this region is by a factor of 10 higher than at other places of the *C. cinerea* genome. One map unit in this region calculates to a DNA sequence length of about 6 kb. In chromosome I with the *A* mating

type locus, we observed a frequency of 20% recombination within a DNA fragment of about 940 kb in length. One map unit corresponds therefore to about 50 kb. Closer to the *A* mating type locus, a ratio of 100 kb to one map unit was defined before (Kües et al. 1992). Within the 50 kb region from the A mating type locus up the *pab1* gene (Kües et al. 1992), the recombination frequencies in three different crosses in this study were 0.4, 0.5 and 1%, respectively. These values are comparable to those observed before in crosses of other *C. cinerea* strains (Day 1960; May and Matzke 1995). On linkage group IV around the *trp1* gene, the ration of the physical to genetic length was reported to be 28 kb to one map unit (Freedman and Pukkila 1997). Of all *C. cinerea* chromosomes, linkage group X with the *B* mating type locus is with a length of 2.2 Mb one of the two smallest chromosomes and linkage group I with the *A* mating type locus with 5.1 Mb the largest (O´Shea et al. 1998). Previous classical mapping gave the impression that the two chromosomes would possibly be the two largest, followed by the chromosome with the *trp1* gene (North 1990). In accordance with our observations, short chromosomes in meiosis of fungi tend to have higher numbers of crossing over per physical length. These crossing overs are however not randomly distributed over the chromosome length. Recombination hotspots and coldspots are common (see recent review by Zickler 2006). Whether the region around the *B* mating type locus presents indeed a recombination hotspot remains to be shown in future studies. Another interesting observation in *C. cinerea* is that for the physical longest chromosome I, only very few molecular markers could be defined with randomly chosen primers unlike to most other chromosomes (Muraguchi et al. 2003, this study). This together with a lower recombination frequency could indicate presence of coldspots on this chromosome.

Further results in this study might substantiate a role of the *B* mating type genes in growth behavior of *C. cinerea*. In both series of crosses performed in course of isogenisation, we obtained collections of strains with wildtype *A* mating type genes that had no uniform growth speed. Usually, the strains with a wildtype *B* mating type grew faster than strains with the *B43mut* mating type. This observation is interesting in view of *B*-mediated mycelial phenotypes. In *S. commune*, a “flat” phenotype caused by activation of the *B* mating type pathway is known from common *A* matings (i.e. matings between strains of alike *A* and different *B* mating type specificities; Raper 1966) as well as from transformation of homokaryons with cloned *B* genes of compatible specificity (Wendland et al. 1995). The “flat” phenotype in *S. commune* is characterized by a lack of aerial mycelium, invasive growth, hyperbranching and a curly irregular hyphal structure (Papazian 1950; Raper and San Antonio 1954; Raper 1966; Jersild et al. 1967; Wendland et al. 1995; Kothe 1996). Whilst an action of *B* mating type genes on mycelial growth and colony appearance is fully accepted in *S. commune*, the situation in *C. cinerea* is less coherent. In their pioneering work on mating type control in dikaryon formation in *C. cinerea*, Swiezynski and Day (1960) did not

observed an obvious *B*-mediated growth phenotype in common *A* matings. With some genetic backgrounds, Muraguchi and Kamada (2002) also had no distinctive mycelial phenotype in common *A* matings. In others, these authors detected (a) dominant genetic factor(s) for such phenotype in common *A* matings but the *B* mating type genes were probably not a direct cause of it. Moreover, when the *B* mating type pathway is activated due to transformations of compatible *B* mating type genes, some but not all *C. cinerea* monokaryons show retarded growth, poor aerial mycelium and changes in mycelial morphology (O´Shea et al. 1997; Kües et al. 2002b). Monokaryon 218 exhibits a particular strong growth retardation reaction upon activation of the *B* mating type pathway (Kües et al. 2002b). In this study, we regularly observed hyphae with zigzag growth in *A42*, *B43mut* F5 and F6 progeny of the JV6 x AmutBmut cross that might contribute to growth retardation. However, we do not know whether this zigzag phenotype is caused directly by the *B43mut* mutation. In the *A3* progenies of generations F5 and F6 of cross 218 x AmutBmut, we observed such zigzag hyphae in clones with the *B43mut* as well as in clones with the wildtype *B1* mating type. Thus, functions other than the activated *B* mating type pathway can give rise to such phenotype.

In the basidiomycete *Pleurotus ostreatus*, several QTLs (quantitative trait loci) for mycelial growth have been described for the monokaryotic mycelium. A number of them are situated close to the *A* mating type locus or close to the two tightly linked *B* mating type subloci. These mating type-linked QTLs have been shown to interact with each other in complex polygenic manner (Larraya et al. 2001, 2002). Our observations on growth differences in progenies that appear to be only different in parts of the mating type chromosomes could indicate that similar complex QTL interactions exist in *C. cinerea*. Most basidiomycete species including *C. cinerea* and *P. ostreatus* are tetrapolar (Kües 2000; Larraya et al. 2001). Linking genes for complex control of vegetative growth of monokaryons in close proximity to the two mating type loci could serve as a positive selection criterion for maintaining the breeding system of a fungus tetrapolar.

3.6. Acknowledgements

We thank Panida Loutchanwoot for many helpful discussions during the progress of this work. PS acknowledges a PhD studentship from the Mahasarakham University, Mahasarakham, Thailand. Work in the laboratory in Göttingen was supported by the DBU (Deutsche Bundesstiftung Umwelt).

3.7. References

Arima, T., and T. Morinaga, 1995 Inheritance of chromosome length polymorphisms in *Coprinus cinereus*. Mycoscience **36:** 9-16.

Arima, T., T. Okida, and T. Moringa, 1996 Behaviour of chromosomes after meiosis in *Coprinus cinereus*. Mycoscience **37:** 111-116.

Ásgeirsdóttir, S. A., J. R. Halsall, and L. A. Casselton, 1997 Expression of two closely linked hydrophobin genes of *Coprinus cinereus* is monokaryon-specific and down-regulated by the *oid-1* mutation. Fungal Genet. Biol. **22:** 54-63.

Badalyan, S. M., E. Polak, M. Aebi, and U. Kües, 2004 Role of peg formation in clamp cell fusion of homobasidiomycete fungi. J. Basic Microbiol. **44:** 167-177.

Binninger, D. M., C. Skrzynia, P. J. Pukkila, and L. A. Casselton, 1987 DNA-mediated transformation of the basidiomycete *Coprinus cinereus*. EMBO J. **6:** 835-840.

Boulianne, R. P., Y. Liu, B. C. Lu, M. Aebi, and U. Kües, 2000 Fruiting body development in *Coprinus cinereus*: regulated expression of two galactins secreted by a non-classical pathway. Microbiology **146:** 1841-1853.

Bottoli, A. P. F., K. Kertesz-Chaloupková, R. P. Boulianne, J. D. Granado, M. Aebi *et al.*, 1999 Rapid isolation of genes from an indexed genomic library of *C. cinereus* in a novel *pab1*$^{+}$ cosmid. J. Microbiol. Meth. **35:** 129-141.

Brown, A. J., and L. A. Casselton, 2001 Mating in mushrooms: increasing the changes but prolonging the affair. Trends Genet. **17:** 393-399.

Casselton, L. A., 1995 Genetics of *Coprinus*, pp. 35-48 in *The Mycota, Vol. II, Genetics and Biotechnology*, edited by U. Kües. Springer-Verlag KG, Berlin, Germany.

Casselton, L. A., and N. S. Olesnicky, 1998 Molecular genetics of mating recognition in basidiomycete fungi. Microbiol. Mol. Biol. Rev. **62:** 55-70.

Casselton, L. A., and M. Riquelme, 2004 Genetics of *Coprinus*, pp. 37-52 *The Mycota, Vol. II., Genetics and Biotechnology*, Ed. 2., edited by U. Kües. Springer-Verlag KG, Berlin, Germany.

Chiu, S. W., and D. Moore, 1990 A mechanism for gill pattern formation in *Coprinus cinereus*. Mycol. Res. **94:** 320-326.

Cummings, W. J., M. Celerin, J. Crodian, L. K. Brunick, and M. E. Zolan, 1999 Insertional mutagenesis in *Coprinus cinereus*: use of a dominant selectable marker to generate tagged, sporulation-defective mutants. Curr. Genet. **36:** 371-382.

Day, P. R., 1960 The structure of the *A* mating type locus in *Coprinus lagopus*. Genetics **45:** 641-650.

Freedman, T., and P. J. Pukkila, 1997 A physical assay for meiotic recombination in *Coprinus cinereus*. Mol. Gen. Genet. **254:** 372-378.

Granado, J. D., K. Kertesz-Chaloupková, M. Aebi, and U. Kües, 1997 Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. Mol. Gen. Genet., **256:** 28-36.

Hiscock, S. J., and U. Kües, 1999 Cellular and molecular mechanisms of sexual incompatibility in plants and fungi. Int. Rev. Cytol. **193:** 165-295.

Ito, Y., T. Fushimi, and S. O. Yanagi, 1998 Discrimination of species and strains of basidiomycete genus *Coprinus* by random amplified polymorphic DNA (RAPD) analysis. Mycoscience **39:** 361-365.

Jersild, R., S. Mishkin, and D. Niederpruem, 1967 Origin and ultrastructure of complex septa in *Schizophyllum commune* development. Arch. Mikrobiol. **57:** 20-32.

Kamada, T., 2002 Molecular genetics of sexual development in the mushroom *Coprinus cinereus*. BioEssays **24:** 449-459.

Kertesz-Chaloupková, K., P. J. Walser, J. D. Granado, M. Aebi, and U. Kües, 1998 Blue light overrides repression of asexual sporulation by mating type genes in the basidiomycete *Coprinus cinereus*. Fungal Genet. Biol. **23:** 95-109.

Kilaru, S., P. J. Hoegger, and U. Kües, 2006 The laccase multi-gene family in *Coprinopsis cinerea* has seventeen different members that divide into distinct subfamilies. Curr. Genet. **50:** 45-60.

Kothe, E., 1996 Tetrapolar fungal mating types: sexes by the thousands. FEMS Microbiol. Rev. **18:** 65-87.

Kothe, E., 2001 Mating-type genes for basidiomycete strain improvement in mushroom farming. Appl. Microbiol. Biotechnol. **56:** 602-612.

Kües, U., 2000 Life history and developmental processes in the basidiomycete *Coprinus cinereus*. Microbiol. Mol. Biol. Rev. **64:** 316-353.

Kües, U., 2002 Sexuelle und asexuelle Fortpflanzung bei einem Pilz mit 12000 Geschlechtern. Vierteljahrsschrift der Naturforschenden Gesellschaft in Zürich **147:** 23-34.

Kües, U., W. V. J. Richardson, A. M. Tymon, E. S. Mutasa, B. Göttgens *et al.*, 1992 The combination of dissimilar alleles of the $A\alpha$ and $A\beta$ gene complexes, whose proteins contain homeo domain motifs, determines sexual development in the mushroom *Coprinus cinereus*. Genes Dev. **6:** 568-577.

Kües, U., J. D. Granado, R. Hermann, R. P. Boulianne, K. Kertesz-Chaloupková *et al.*, 1998 The *A* mating type and blue light regulate all known differentiation processes in the basidiomycete *Coprinus cinereus*. Mol. Gen. Genet. **260:** 81-91.

Kües, U., M. J. Klaus, E. Polak, and M. Aebi, 2001 Multiple cotransformation in *Coprinus cinereus*. Fungal Genet. Newsl. **48:** 32-34.

Kües, U., E. Polak, A. P. F. Bottoli, M. Hollenstein, P. J. Walser *et al.*, 2002a Vegetative development in *Coprinus cinereus*, pp. 133-163 in *Molecular Biology of Fungal Development*, edited by H.D. Osiewacz. Marcel Dekker, New York.

Kües, U., P. J. Walser, M. J. Klaus, and M. Aebi, 2002b Influence of activated *A* and *B* mating-type pathways on developmental processes in the basidiomycete *Coprinus cinereus*. Mol. Genet. Genomics **268:** 262-271.

Kües, U., M. Künzler, A. P. F. Bottoli, P. J. Walser, J. D. Granado *et al.*, 2004 Mushroom development in higher basidiomycetes; implications for human and animal health, pp. 431-470 in *Fungi in Human and Animal Health*, edited by R.K.S. Kushwaha. Scientific Publishers, Jodhpur, India.

Larraya, L. M., G. Pérez, I. Iribarren, J. A. Blanco, M. Alfonso *et al.*, 2001 Relatioship between monokaryotic growth rate and mating type in the edible basidiomycete *Pleurotus ostreatus*. Appl. Env. Microbiol. **67:** 3385-3390.

Larraya, L. M., E. Idareta, D. Arana, E. M. Ritter, A. G. Pisabarro *et al.*, 2002 Quantitative trait loci controlling vegetative growth rate in the edible basidiomycete *Pleurotus ostreatus*. Appl. Env. Microbiol. **68:** 1109-1114.

Leslie, J. F., 1981. Inbreeding for isogeneity by backcrossing to a fixed parent in haploid and diploid eukaryotes. Genet. Res. **37:** 239-252.

Liu, Y., 2001. *Fruiting Body Initiation in the Basidiomycete Coprinus cinereus*. PhD thesis, ETH Zurich, Zurich, Switzerland.

Liu, Y., J. Granado, E. Polak, and U. Kües, 2001 Crosses with *Amut Bmut* homokaryons of *Coprinus cinereus*. Fungal Genet. Newsl. **46:** 16-20.

Liu, Y., P. Srivilai, S. Loos, M. Aebi, and U. Kües, 2006 An essential gene for fruiting body initiation in the basidiomycete *Coprinopsis cinerea* is homologous to bacterial cyclopropane fatty acid synthase genes. Genetics **172:** 873-884.

Lu, B. C., N. Gallo, and U. Kües, 2003 White-cap mutants and meiotic apoptosis in the fungus *Coprinus cinereus*. Fungal Genet. Biol. **39:** 82-93.

May, G., and E. Matzke, 1995 Recombination and variation at the *A* mating-type of *Coprinus cinereus*. Mol. Biol. Evol. **12:** 794-802.

May, G., and J. W. Taylor, 1988 Patterns of mating and mitochondrial DNA inheritance in the agaric basidiomycete *Coprinus cinereus*. Genetics **118:** 213-220.

Moore, D., 1981 Developmental genetics of *Coprinus cinereus*: Genetic evidence that carpophores and sclerotia share a common pathway of initiation. Curr. Genet. **3:** 145-150.

Muraguchi, H., and T. Kamada, 2002 A distinctive phenotype in the common-*A* heterokaryon of *Coprinus cinereus*. Mycoscience **43:** 77-80.

Muraguchi, H., T. Takemaru, and T. Kamada, 1999 Isolation and characterization of developmental variants in fruiting using a homokaryotic fruiting strain of *Coprinus cinereus*. Mycoscience **40:** 227-233.

Muraguchi, H., Y. Ito, T. Kamada, and S. O. Yanagi, 2003 A linkage map of the basidiomycete *Coprinus cinereus* based on random amplified polymorphic DNAs and restriction fragment length polymorphisms. Fungal Genet. Biol. **240:** 93-102.

North, J., 1990 Linkage map of *Coprinus cinereus* Schaeff. Ex Fr. S.F.Gray, Ink cap. N=12, pp. 3.70-3.81 in *Genetic Maps: Locus Maps of Complex Genomes*, edited by S. J. O'Brien. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

O´Shea, S. F., P. T. Chaure, J. R. Halsall, N. S. Olesnicky, A. Leibbrant *et al.*, 1998 A large pheromone and receptor gene complex determines multiple *B* mating specificities in *Coprinus cinereus*. Genetics **148:** 1081-1090.

Papazian, H. P., 1950 Physiology of the incompatibility factors in *Schizophyllum commune*. Bot. Gaz. **112:** 143-163.

Polak, E., M. Aebi, and U. Kües, 2001 Morphological variations in oidium formation in the basidiomycete *Coprinus cinereus*. Mycol. Res. **105:** 603-610.

Pukkila, P. J., 1993 Methods of genetic manipulation in *Coprinus cinereus*, pp. 249-264 in *Genetics and Breeding of Edible Mushrooms*, edited by S.T. Chang, J.A. Buswell and P.G. Miles.Gordon and Breach Science Publishers, Y-Parc, Switzerland.

Pukkila, P. J., 1994. Meiosis in mycelial fungi, pp. 267-282 in *The Mycota, Vol. I., Growth, Differentiation and Sexuality*, edited by J. G. H. Wessels and F. Meinhardt. Springer-Verlag, New York.

Pukkila, P. J., and B. C. Lu, 1985 Silver staining of meiotic chromosomes in the fungus *Coprinus cinereus*. Chromosoma **91:** 108-112.

Raper, J. R., 1966 *Genetics of Sexuality in Higher Fungi*. The Ronald Press, New York.

Raper, J. R., and R. M. Hoffman, 1974 *Schizophyllum commune*, pp. 597-626 in *Handbook of Genetics:Bacteria, Bacteriophages, and Fungi*, edited by R. C. King. Plenum Press, New York.

Raper, J. R., and G. S. Krongelb, 1958 Genetic and environmental aspects of fruiting in *Schizophyllum commune* Fr. Mycologia **50:** 707-740.

Raper, J. R., and M. T. Oettinger, 1962 Anomalous segregation of incompatibility factors in *Schizophyllum commune*. Rev. Biol. (Lisbon) **3:** 205-221.

Raper, J. R., and J. P. San Antonio, 1954 Heterokaryotic mutagenesis in Hymenomycetes. I. Heterokaryon in *Schizophyllum commune*. Am. J. Bot. **41:** 69-86.

Srivilai, P., W. Chaisaena, and U. Kües, 2005 Genetic analysis of *Coprinopsis cinerea* mutants with defects in fruiting body development in *VI Genetics and Cellular Biology of Basidiomycetes*, edited by G. Pisabarro and L. Ramírez. University of Publica de Navarra, Pamplona, Spain, in press.

Swamy, S., I. Uno, and T. Ishikawa, 1984 Morphogenetic effects of mutations at the *A* and *B* incompatibility factors of *Coprinus cinereus*. J. Gen. Microbiol. **130:** 3219-3224.

Swiezynski, K. M., and P. R. Day, 1960 Heterokaryon formation in *Coprinus lagopus*. Genet. Res. (Camb.) **1:** 114-128.

Takemaru, T., 1982 Genetic map of *Coprinus macrorhizus*, pp. 355-356 in *Experimental Methods in Microbial Genetics*, edited by T. Ishikawa. Kyoritsu, Tokyo, Japan.

Tymon, A. M., U. Kües, W. V. J. Richardson, and L. A. Casselton, 1992 A fungal mating type protein that regulates sexual and asexual development contains a POU-related domain. EMBO J. **11:** 1805-1813.

Uno, I., and T. Ishikawa, 1971 Chemical and genetic control of induction of monokaryotic fruiting bodies in *Coprinus macrorhizus*. Mol. Gen. Genet. **113:** 228-239.

Velagapudi, R., 2006 *Extracellular Matrix Proteins in Growth and Fruiting Body Development of Straw and Wood Degrading Basisidiomycetes*. PhD thesis, Georg-August-University of Göttingen, Göttingen, Germany.

Walser, P. J., M. Hollenstein, M. J. Klaus, and U. Kües, 2001 Genetic analysis of basidiomycete fungi, pp. 59-90 in *Molecular and Cellular Biology of Filamentous Fungi: A Practical Approach*, edited by N. J. Talbot. IRL Press, Oxford.

Walser, P. J., R. Velagapudi, M. Aebi, and U. Kües, 2003 Extracellular matrix proteins in mushroom development. Recent Res. Dev. Microbiol. **7:** 381-415.

Wendland, J., L. J. Vaillancourt, J. Hegner, K. B. Lengeler, K. J. Laddison *et al.*, 1995 The mating-type *Bα1* of *Schizophyllum commune* contains a pheromone receptor gene and putative pheromone genes. EMBO J. **14:** 5271-5278.

Wessels, J. G. H., 1999 Fungi in their own rights. Fungal Genet. Biol. **27:** 134-145.

Zickler, D., 2006 Meiosis in mycelial fungi, pp. 415-438 in *The Mycota, Vol. I., Growth, Differentiation and Sexuality*, edited by U. Kües and R. Fischer. Springer, Berlin, Germany.

Zolan, M. E., 1995 Chromosome-length polymorphism in fungi. Microbiol. Rev. **59:** 686-698.

Zolan, M. E., and P. J. Pukkila, 1986 Inheritance of DNA methylation in *Coprinus cinereus*. Mol. Cell. Biol. **6:** 405-409.

Zolan, M. E., J. R. Crittenden, N. K. Heyler, and L. C. Seitz, 1992 Efficient isolation and mapping of *rad* genes of the fungus *Coprinus cinereus* using chromosome-specific libraries. Nucleic Acids Res. **20:** 3993-3999.

Zolan, M. E., N. K. Heyler, and M. A. Ramesh, 1993 Gene mapping using marker chromosomes in *Coprinus cinereus*, pp. 31-35 in *Industrial Microorganisms: Basic and Applied Molecular Genetics*, edited by R. H. Baltz, G. D. Hegemann and P. L. Skatrud. American Society for Microbiology, Washington DC.

Zolan, M. E., N. K. Heyler, and N. Y. Stassen, 1994 Inheritance of chromosome-length polymorphisms in *Coprinus cinereus*. Genetics **137:** 87-94.

CHAPTER 4

An essential gene for fruiting body initiation in the basidiomycete *Coprinopsis cinerea* is homologous to bacterial cyclopropane fatty acid synthase genes

Liu, Y., Srivilai, P., Loos, S., Aebi, M., and Kües, U.

Published in: Liu, Y., **Srivilai, P.,** Loos, S., Aebi, M., and Kües, U. 2006. An essential gene for fruiting body initiation in the basidiomycete *Coprinopsis cinerea* is homologous to bacterial cyclopropane fatty acid synthase genes. Genetics, **172**: 873-884

This manuscript is based on chapter 4 in thesis of Yi Lui (2001), performed at the ETH Zurich in Switzerland. To this study, I contributed the classical genetics study of resolving the number of mutations in fruiting body development present in *C. cinerea* mutant 6-031 (paragraph 4.4.1). I selected the *cfs1* mutant OU3-1 from crosses of monokaryon PS001-1 and mutant 6-031 and transformed it with various DNA fragments with the *C. cinerea cfs1* gene subcloned by Yi Liu and observed the behaviour in fruiting of the transformants (paragraph 4.4.5).

4.1. Abstract

The self-compatible *Coprinopsis cinerea* homokaryon AmutBmut produces fruiting bodies without prior mating to another strain. Early stages of fruiting body development include the dark-dependent formation of primary hyphal knots and the light-induced transition from primary hyphal knots to the more compact secondary hyphal knots. The AmutBmut UV mutant 6-031 forms primary hyphal knots, but development arrests at the transition state. Genetic analysis indicates that this phenotype is caused by a single recessive defective gene. Gene *cfs1* was isolated from a cosmid library by mutant complementation. A normal primordia phenotype was achieved when *cfs1* was embedded at both sides in at least 4.0 kb of native flanking DNA. Truncations of the flanking DNA lead to reduction in transformation frequencies and faults in primordia formation, suggesting that the gene is also needed at later stages of development. *cfs1* encodes a protein highly similar to cyclopropane fatty acid synthases, a class of enzymes shown in prokaryotes and recently in a plant to convert membrane-bound unsaturated fatty acids into cyclopropane fatty acids. In *C. cinerea* 6-031, the mutant *cfs1* allele carries a T to G transversion, leading to an amino acid substitution (Y441D) in a domain suggested to be involved in the catalytic function of the protein and/or membrane interaction.

4.2. Introduction

The heterothallic fungus *Coprinopsis cinerea* serves as a model organism to study fruiting body development in higher basidiomycetes. Fruiting bodies normally develop on the dikaryon (Kües 2000). However, the presence of two genetically distinct nuclei in the dikaryotic mycelium is a major drawback to perform genetic analysis on fruiting body development. The self-compatible homokaryon AmutBmut, having specific mutations in both mating-type loci (*A43mut* and *B43mut*) shows characteristics typical to the dikaryon, for example formation of fused clamp cells at hyphal septa. It also gives rise to fruiting bodies without prior mating to another strain (Swamy et al. 1984; Walser et al. 2003). This special feature together with the ability to form unicellular haploid spores (oidia) provides us an easy accessible genetic system (Kües et al. 2004). A series of developmental mutants have been generated from strain AmutBmut by UV- and REMI-mutagenesis (Granado et al. 1997; Lu et al. 2003; Kües et al., unpublished), from which genes can be isolated.

The development of fruiting bodies is a highly organized process, which requires the coordination between genetic, environmental and physiological factors (Kües 2000). In the dark, upon nutritional depletion, single hyphae locally undergo intense branching to form microscopic primary hyphal knots. When kept in the dark, these develop into multicellular pigmented resting bodies called sclerotia (Kües et al. 2002a). Following a light signal, radial growth of primary hyphal knots and hyphal interaction lead to the formation of compact hyphal aggregates, secondary hyphal knots, which are specific fruiting body initials. Cellular differentiation within the secondary hyphal knot results in the formation of cap and stipe tissues. Such differentiated structures are termed primordia (Boulianne et al. 2000; Kües 2000; Walser et al. 2003). In the primordium cap, induced by a further light signal, karyogamy occurs in specialized cells (basidia). Karyogamy is directly followed by meiosis. In parallel, the stipe elongates and the cap expands, giving rise to a fully developed fruiting body (Moore et al. 1979; Kües 2000).

So far, little is known about the genetic determinants that act in fruiting body initiation and formation. Induction of primary hyphal knots and the morphological transition from primary into secondary hyphal knots were shown to be regulated by the *A* and the *B* mating type genes (Kües et al. 1998, 2002b). A gene *pcc1*, encoding an HMG-box transcription factor, likely acts downstream of the *A* mating-type gene products and appears to negatively regulate fruiting body initiation (Murata et al. 1998). Onset of expression of two genes encoding fruiting body specific galectins (β-galactoside sugar binding lectins) correlates with the formation of primary and secondary hyphal knots, respectively, and continues during primordia development (Boulianne et al. 2000; Bertossa et al. 2004). In addition, three genes have been identified that act in cap and stipe tissue formation and stipe elongation, respectively (Muraguchi and Kamada 1998, 2000; Arima et al. 2004).

Within our mutant collection derived from homokaryon AmutBmut, we identified two groups of mutants whose defects link to fruiting body initiation. Members of one group are termed *pkn* (= primary knotless) mutants, because they do not form any primary hyphal knot in the dark. The other group of mutants is arrested at the transition from primary to secondary hyphal knots. Therefore, they are called *skn* (= secondary knotless) mutants (Kües et al., unpublished). In this study, we isolated a gene that complemented the defect of fruiting body initiation in the *skn1* UV-mutant 6-031. The predicted gene product is highly homologous to cyclopropane fatty acid synthases, a class of enzymes characterized before in bacteria and recently also in a plant.

4.3. Materials and methods

4.3.1. Fungal strains, culture conditions and transformation

C. cinerea strains were standardly grown at 37°C on YMG/T complete medium and minimal medium (Granado et al. 1997) supplemented with p-aminobenzoate (PABA, 5 mg/l) when required. Strain 6-031 (*A43mut, B43mut, pab1-1, skn1*) is a fruiting body initiation mutant generated from homokaryon AmutBmut (*A43mut, B43mut, pab1-1*) by UV-mutagenesis (Kües et al., unpublished). Monokaryon JV6 (*A42, B42*) unrelated to homokaryon AmutBmut and the AmutBmut co-isogenic monokaryons PS001-1 (*A42, B42*) and PS002-1 (*A3, B1*) were used in crosses (Kertesz-Chaloupková et al. 1998; Srivilai et al., in preparation). Matings were performed on YMG/T plates by placing two mycelial blocks of inoculum 5 mm apart. For growth and induction of fruiting bodies, mating plates were incubated in standard fruiting conditions (Granado et al. 1997). Randomly isolated basidiospores were germinated on YMG/T medium at 37°C. Progenies of crosses were analysed on minimal media for PABA-auxotrophy. Presence of unfused and fused clamp cells, indicators of activated *A* and *B* mating-type pathways, respectively (Kües 2000), was determined by microscopy. Monokaryon JV6 served to confirm mating types in *A43mut, B43mut* progenies from crosses with PS001-1 and PS002-1 that subsequently were submitted to fruiting tests. Frequencies of phenotypic distributions in *A43mut, B43mut* progenies were tested by a Chi-square method. A *skn1+, mat+ , bad* clone (PS-Mu1-3) and a *skn1+, mat, bad* clone (PS-Mu1-1) with the A42, B42 progeny of cross 6-031 x PS001-1 (defined by crosses with monokaryon PG78) were identified through mating with mutant 6-031. Mating of these two strains with A43mut, B43mut clones of the progeny 6-031 x PS001-1 that did not imitate fruiting identified homokaryon OU3-1 (*A43mut, B43mut, pab1-1, skn1*).

The F1 progeny of cross 6-031 x JV6 was randomly analyzed for fruiting ability by individually inoculating clones on YMG/T agar, growing them for 4 days at 37°C in the dark and subsequently transferring them to standard fruiting conditions. Dikaryons amongst the clones were

identified by light inducing oidia production, germinating the spores on YMG/T agar and analyzing pab-auxotrophy on minimal medium. For oidia induction of dikaryons, mutant 6-031 and other *A43mut, B43mut* strains, dark grown cultures were exposed to light for two days (Kertesz-Chaloupková et al. 1998). The number of oidia per plate was determined by a spectrophotometer (Polak 1999).

DNA transformation was performed as described (Granado et al. 1997). For selecting pab-prototrophs in cotransformations, 1 μg of plasmid pPAB1-2 (Granado et al. 1997) was added. Upon germination on regeneration agar, transformants were individually transferred onto minimal medium for further growth. Subsequently, three or four individual transformants were inoculated on YMG/T agar per single Petri dish and grown in the dark at 37°C for 2 days to a colony size of 3-3.5 cm in diameter. To induce fruiting, plates were moved for 2 weeks to standard fruiting conditions. The number and size of primordia per transformant were scored. A small piece of gill tissue from primordia developed upon transformation with cosmid 40-5A was spread and nuclei in basidia stained with hematoxylin (Lu and Raju 1970).

4.3.2. DNA and RNA techniques

An indexed genomic cosmid library derived from homokaryon AmutBmut was transformed into mutant 6-031 and screened for cosmids that were able to restore fruiting ability in this strain, following a SIB-selection procedure. The *pab1*$^{+}$ wild-type gene of *C. cinerea* present in the cosmid backbone was used as a selection marker. Cosmid DNAs from 60 pools of each 96-well microtiter dish-arranged *E. coli* clones, and from subpools and individual clones of microtiter dish 40 were isolated (Bottoli et al. 1999).

Cloning was performed by standard methods (Sambrook et al. 1989). Plasmids were propagated in *E. coli* strain XL1-Blue (Stratagene). Derivative pSphA of cosmid 40-5A is a ligation product between a 16 kb *Sph*I fragment (13 kb genomic DNA + 3 kb cosmid backbone) and a 7.5 kb *Sph*I fragment (2 kb genomic DNA + 5.5 kb cosmid backbone) in their natural order. *Not*I fragments of cosmid 40-5A were cloned into the *Not*I site of pBC SK (+) (Stratagene). Plasmids pNotB5 and pNotB7 contained the same DNA insert but in opposite orientation. The insert in pNotB5 was sequenced on both strands by primer walking (Microsynth, Balgach, Switzerland). Sequences were assembled with program DNASTAR and analyzed with OMIGA 2.0, BLAST (NCBI). The whole sequence but 32 bp originating from the linker of the cosmid was submitted to GenBank (AF338438).

pNotB5 and pNotB7 gave rise to the following pBC SK (+) subclones: p5SmaCS and p5BamCS (used in Northern analysis) carry gene *arf1* on a 1.4 kb *Not*I-*Sma*I and a 3.5 kb *Not*I-*Bam*HI fragment, respectively. p5EcoCS and p5XbaCS contain *arf1* and a truncated *cfs1* gene on a

3.8 kb *Not*I-*Eco*RI and a 5.5 *Not*I-*Xba*I fragment, respectively. p5SpeCS includes both *arf1* and *cfs1* on a 7 kb *Spe*I fragment whereas *cfs1* is truncated in p5KpnCS on the shorter 4.4 kb *Not*I-*Kpn*I fragment. p7XbaCS carries truncated *cfs1* and *kin1* copies on a 5 kb *Xba*I-*Not*I fragment. p7SpeCS contains a truncated *kin1* on a 3.5 kb *Spe*I-*Not*I fragment. Subclones constructed in pBluescript KS (-) (Stratagene) were as follows: pPvu8.5 contains a 8.5 kb *Pvu*II fragment covering the complete *cfs1* gene and the 3´ end of *kin1*. pBam3.5 and pSmaSpe5.5 carry *cfs1* on a 3.5 kb *Bam*HI and a 5.5 kb *Sma*I-*Spe*I insert, respectively. pEco4.4 contains truncated *cfs1* and *kin1* copies on a 4.4 kb *Eco*RI fragment. Furthermore, the 8.5 kb *Pvu*II fragment, 3.5 kb *Bam*HI fragment and 4.4 kb *Eco*RI fragment were also cloned into pPAB1-2 containing the *C. cinerea pab1*$^{+}$ gene, resulting in pPvu8.5-pab, pBam3.5-pab and pEco4.4-pab, respectively.

A cut-and-shut strategy using *Nco*I and plasmid pNotB7 resulted in p7NcoCSΔ*cfs1* with a deletion in *cfs1* (Δbp 5296-5476). Similarly, p5BstCSΔ *kin1* with a deletion in *kin1* (Δbp 8502-8619) were created from plasmid p5NotB5 by using *Bst*EII. An *Aat*II deletion (Δbp 6567-6803) in p5SpeCS yielded p5SpeCS *gtl*. A *Nru*I deletion in *arf1* (Δbp 676-972) in p5SmaCS gave rise to p5SmaCSΔ*arf*, from which the *Sma*I insert was cloned into pSamSpe5.5 to generate p5SpeCSΔ*arf*. The insertion of a 3.5 kb *Spe*I fragment from pNotB7 at the *Spe*I site in p5SpeCSΔ*arf* resulted in pNotB5Δ*arf*. The T to G transversion found in the *cfs1* allele of mutant 6-031 was introduced into plasmid p5SpeCS by exchanging a 1 kb PCR amplified *Stu*I–*Nde*I fragment with the wild-type sequence, yielding p5SpeCS/6-031. pNotB5/6-031 distinguishes from pNotB5 by the same T to G transversion.

Genomic DNA of *C. cinerea* strains was isolated from powdered lyophilized mycelium (Zolan and Pukkila 1986). Two overlapping fragments containing the *cfs1* allele of mutant 6-031 were 6x independently amplified from genomic DNA with specific primers (a 3.1 kb fragment using primers 5´TCAAGTCGGGTCGGTAGAAG3´ and 5´TTTGTTTCGGAGCTTGACTG3´ and a 1.1 kb fragment using primers 5´GGACGCTTCAAGATTAGATC3´ and 5´CTCTGAAGGAATCGCTCTTG3´) and sequenced using a ABI PRISM DNA Sequencing Kit and a Model 373A DNA sequencer (Perkin-Elmer). Sequences of PCR products separately amplified with the same primer set were identical. Presence of the same sequence in p5SpeCS/6-031 has also been verified by sequencing.

Southern blot analysis was performed with 10 μg of genomic DNA per sample following basic protocols (Sambrook et al. 1989). Total RNA of strain AmutBmut was extracted with a guanidinium isothiocyanate procedure (Chomczynski and Sacchi 1987) from powdered lyophilized *C. cinerea* mycelia or tissues of different fruiting stages. poly(A)$^{+}$ RNA was isolated with the Oligotex mRNA Midi kit (Qiagen). Per sample, 10 μg of total RNA or 2.5 μg of poly(A)$^{+}$ RNA

were used for Northern blot analysis (Sambrook et al. 1989). Hybridization signals in Southern and Northern blot analysis were produced with DNA fragments labeled with [α-^{32}P]dCTP by random primed DNA labeling (Boehringer Mannheim).

The 5´ and 3´ cDNA ends of the *cfs1* gene were determined with the 5´/3´ RACE Kit of Roche Molecular Biochemicals following the instructions of the manufacturer. poly(A)$^+$ RNA from 5 mm-sized primordia was used for cDNA synthesis. In the 5´ RACE, a *cfs1* specific primer sp1 (5´ACAATGCACAGGAGTACATC3´) was employed to synthesize the first strand cDNA. Two *cfs1* specific primers, sp2 (5´GCAATGGCATTGAGTCGAG3´) and sp3 (5´TAGACGATAGGGT-CATCTCC3´), were applied in subsequent PCR reactions. In the 3´ RACE, two *cfs1* specific primers, sp4 (5´GATTTTGCCCTCAAGCCAC3´) and sp5 (5´CAATTCGAGCCTGCCCAG3´) were used. RACE products were cloned into pBluescript KS (-) by T/A cloning (Marchuk et al. 1991) and sequenced with a Model 373A DNA sequencer. The full coding length of the *cfs1* cDNA was obtained by PCR, using the two primers cfsATG (5´ATGCCGGCCCACCACCACCCTTC3´) and cfsREV (5´CGCCGAGGCCGCCGTGTAAACAC3´). For sequencing, the PCR product was cloned into the *Eco*RV site in the β-galactosidase gene of pBluescript KS (-) via T/A cloning, resulting in construct pYL28 having the *cfs1* cDNA inserted in frame to the β-galactosidase gene.

4.3.3. Computer analysis of protein sequence

Proteomic tools provided by ExPaSy Molecular Biology Server (Swiss Institute of Bioinformatics, Geneva) were used to perform protein pattern and profile searches (InterPro), transmembrane region detection (TMpred and TMHMM) and secondary structure predication (PSA and PSIpred). Hydrophilicity profile was calculated with Goldman/Engelman/Steitz parameters in OMIGA 2.0.

4.4. Results

4.4.1. Morphological and genetic analysis of UV-mutant 6-031

UV-mutant 6-031 has a growth rate (8 mm/day on YMG/T agar at 37°C) and a mycelial morphology indistinguishable from its progenitor strain AmutBmut. Like homokaryon AmutBmut (Kertesz-Chaloupková et al. 1998; Badalyan et al. 2004), the mutant forms fused clamp cells at the hyphal septa and produces ca. 10^9 oidia/plate in a light-dependent manner, indicating that mating-type functions in mutant 6-031 are not affected (Kües 2000). Mutant 6-031 forms primary hyphal knots in the dark that mature into sclerotia when cultures are further kept in the dark (not shown). However, primary hyphal knots do not develop into secondary hyphal knots in a 12 h light/12 h

dark regime at 25°C with 90% humidity (standard fruiting conditions), suggesting that mutant 6-031 has a specific defect in fruiting body initiation (*skn1*). As the wildtype, mutant 6-031 is also not able to initiate fruiting body development in constant dark, constant light or at other temperatures, or on minimal medium.

Crosses between strain 6-031 and the AmutBmut co-isogenic monokaryons PS001-1 (*A42*, *B42*) and PS001-2 (*A3*, *B1*) gave rise to mature fruiting bodies, indicating that mutant 6-031 carries a recessive defect in fruiting body initiation. Self-compatible *A43mut*, *B43mut* descendants of the crosses were subjected to a fruiting test (49 clones from cross PS001-1 x 6-031; 64 clones from cross PS002-1 x 6-031). 65% of the *A43mut*, *B43mut* progeny of cross PS001-1 x 6-031 (i.e. 32 clones) and 59% of cross PS002-1 x 6-031 *A43mut*, *B43mut* progeny (i.e. 38 clones) did not form secondary hyphal knots, suggesting that there is one defect in fruiting body initiation in mutant 6-031 (*skn1*) that is not linked to the mating type genes ($p<0.05$; for comparison of background failure in fruiting initiation, 6% and 5% of *A43mut*, *B43mut* progenies of parallel crosses PS001-1 x AmutBmut and PS002-1 x AmutBmut did not initiate fruiting; Srivilai et al., in preparation). 10 clones (20%) and 12 clones (19%) of the *A43mut*, *B43mut* progenies of crosses PS001-1 x 6-031 and PS002-1 x 6-031, respectively, arrested development during primordia formation, indicating that mutant 6-031 has a second unlinked recessive defect in primordia maturation (*mat*; $p<0.05$). The remaining clones split into two further groups, one with white mushrooms by a failure in basidiospore formation (2 clones, i.e. 4% of *A43mut, B43mut* progeny of cross PS001-1; 7 clones, i.e. 11% of *A43mut, B43mut* progeny of cross PS001-1) and one with mushrooms carrying mature black basidiospores (5 clones, i.e. 11% of *A43mut, B43mut* progeny of cross PS001-1; 7 clones, i.e. 11% of *A43mut*, *B43mut* progeny of cross PS001-1). Therefore, a third unlinked recessive gene is present in mutant 6-031 that acts in basidiospore formation (*bad*; $p<0.05$).

4.4.2. Identification of a cosmid able to restore fruiting body initiation in mutant 6-031

A genomic library of *C. cinerea* monokaryon AmutBmut (Bottoli et al. 1999) was employed to isolate DNA sequences that restored the fruiting initiation in mutant 6-031. In a first round of transformations using pools of 96 different cosmids, one transformant in a total number of 7948 (equivalent to the analysis of ca. 45% of the entire library) formed primordia up to a size of 5-8 mm (Fig. 1). In subsequent transformations dividing the positive pool 40 into subpools, 12 out of 208 transformants of subpool 40-5 developed primordia, and in the final round using individual cosmids, 27 out of 45 tested transformants of cosmid 40-5A. Basidia within these primordia had either two distinct nuclei at the prekaryogamy stage (Fig. 1) or no nucleus (data not shown).

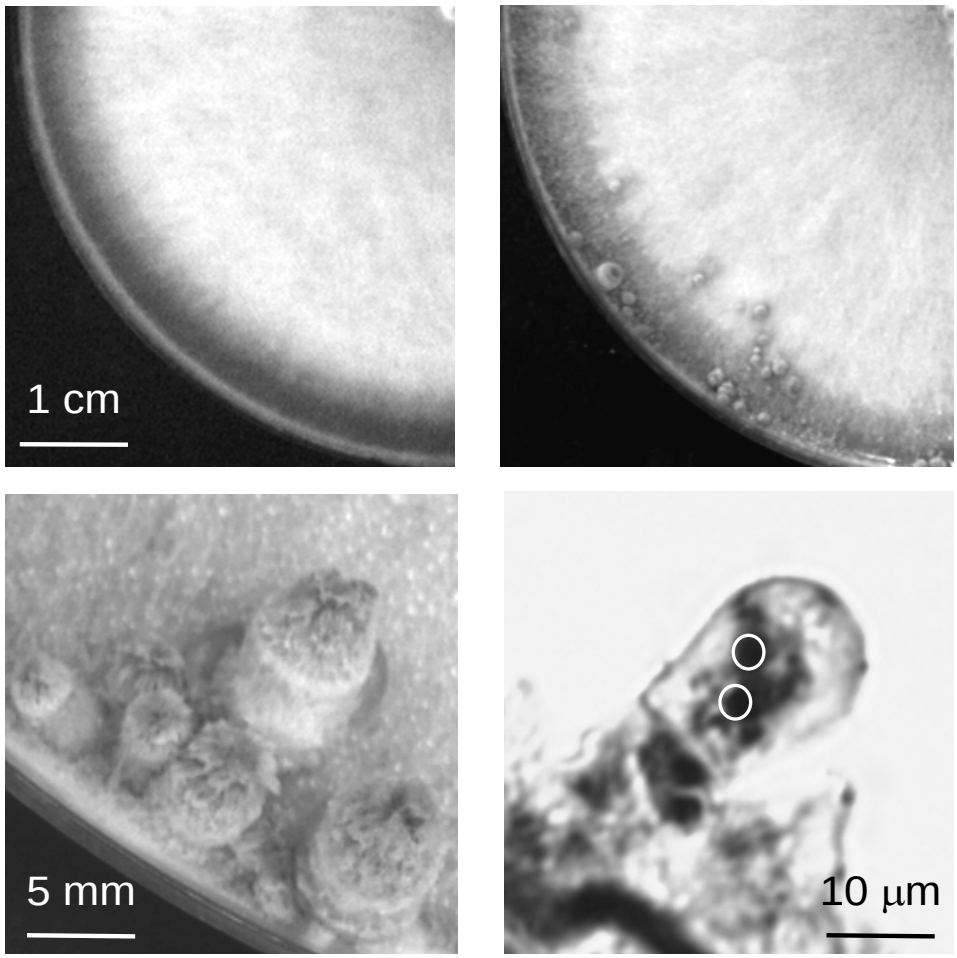


Figure 1. Cosmid 40-5A restores the defect in fruiting initiation in the *Coprinopsis cinerea* mutant 6-031 upon transformation. Mycelial morphology of mutant 6-031 before transformation (top left) and primordium formation after transformation (top right). Primordia of transformants are shown enlarged at the bottom left. Their basidia (bottom right) are in a stage of prekaryogamy, as indicated by the presence of the two nuclei and their positions within the basidia (Kües 2000).

4.4.3. Transformation activities of subclones derived from cosmid 40-5A in strain 6-031

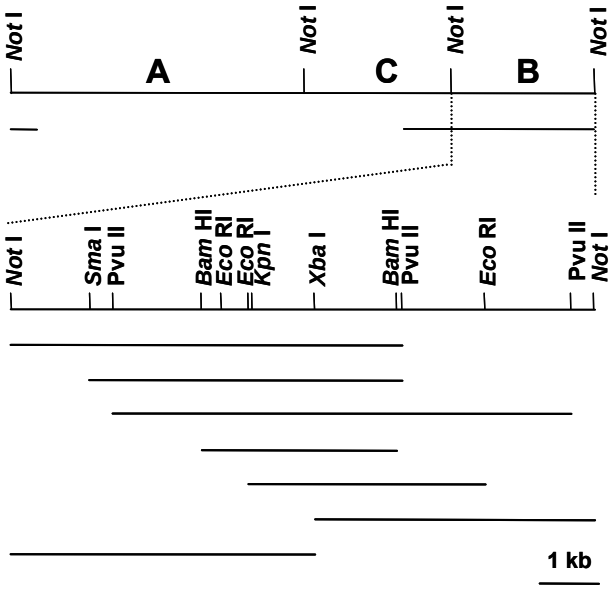
Cosmid 40-5A with a 40 kb sized insert of *C. cinerea* genomic DNA was digested with various restriction enzymes, and digestion mixtures were transformed into mutant 6-031. Digestions with *Bam*HI, *Not*I, *Pvu*II or *Sph*I still allowed initiation of fruiting body development in part of the transformants, unlike a number of other enzymes (*Eco*RI, *Eco*RV, *Kpn*I, *Pst*I or *Xho*I). *Not*I divides cosmid 40-5A into three *C. cinerea* genomic DNA fragments (20, 10.5 and 8.9 kb, Fig. 2A) plus an extra fragment representing the cosmid backbone (8.9 kb). *Not*I was chosen to construct pBC SK(+) subclones, which were cotransformed with plasmid pPAB1-2 into mutant 6-031. Plasmids pNotB5 and pNotB7 containing the same 10.5 kb insert fragment (*Not*I-B), restored fruiting body initiation (Fig. 2A, B). Some subclones of this *C. cinerea* genomic fragment (p5SpeCS, pSmaSpe5.5, pPvu8.5 and pBam3.5) were also active in fruiting body initiation. However, we noticed

quantitative and qualitative variations in transformation activities, related to the length of transformed DNA fragments (Fig. 2A, B). The most effective constructs were cosmid 40-5A and its deletion derivative pSphA carrying the *Not*I-B fragment together with flanking DNA regions. Usually, 20-30% transformants of these cosmids initiated fruiting body development and developed primordia up to 5-8 mm in size. Four plasmids (pNotB5, pNotB7, p5SpeCS and pSmaSpe5.5, containing a common 5.5 kb sequence) induced fruiting body initiation in 5-10% of the transformants, but the primordia formed were of a maximal size of only 2-5 mm (Fig. 2A, B). Normal cap and stipe differentiation were observed in these primordia (Fig. 2B). The reduction in percentage of transformants initiating fruiting might relate to the fact that cosmids 40-5A and pSphA carry the *pab1*$^+$ selection marker, whereas the pBC SK(+) and pBluescript KS(-) constructs needed to be cotransformed with the *pab1*$^+$ containing plasmid pPAB1-2. In contrast, this difference in the transformation procedure cannot account for the less developed primordia obtained when transforming with plasmids pNotB5, pNotB7, p5SpeCS and pSmaSpe5.5. Moreover, upon cotransformation of plasmids pPAB1-2 and pPvu8.5 (having a 5.2 kb *C. cinerea* sequence in common with the former four plasmids), only 1-2% of transformants developed primordia, which were malformed and maximal 2-3 mm in size (Fig. 2A, B). In these primordia, the internal pileus trama tissue was missing (Fig. 2B). Plasmid pBam3.5 with a 3.5 kb *Bam*HI fragment was the smallest construct regularly active in cotransformation, with 0.2-1% of transformants initiating fruiting but development arrested shortly after secondary hyphal knot formation at a size of about 1 mm (Fig. 2A, B). When solely transforming constructs pPvu8.5-pab and pBam3.5-pab, containing the *pab1*$^+$ selection marker in addition to the 8.5 kb *Pvu*II fragment or the 3.5 kb *Bam*HI fragment, neither the transformation efficiency increased nor the primordia development improved in positive transformants obtained (not shown). The data suggest that the observed differences in transformation efficiency and degree of primordia maturation obtained with different *C. cinerea* fragments are not simply a result of variations in the transformation procedure.

The 3.5 kb *Bam*HI fragment present in pBam3.5 originated from the central region of the 10.5 kb *Not*I-B fragment (Fig. 2A). In a total of 498 clones obtained from cotransformation of pPAB1-2 and pEco4.4, a partial overlapping 4.4 kb *Eco*RI fragment only gave rise to 1 transformant (0.2%) able to initiate fruiting (Fig. 2A). Two more transformants with primordia out of 200 tested clones were obtained from transforming mutant 6-031 with plasmid pEco4.4-pab, containing both the 4.4 kb *Eco*RI fragment and the *C. cinerea pab1*$^+$ gene. Interestingly, primordia of these three transformants developed to a size and shape comparable to that of pNotB5 (not shown). Other plasmids carrying *C. cinerea* inserts either from the flanking regions of the 3.5 kb *Bam*HI fragment or inserts splitting this fragment in half were all negative in transformation (Fig. 2A).

Figure 2

A



Construct	Positive/total transformants I	Positive/total transformants II	Maximal primordium size
40-5A	177 / 693	14 / 55	5-8 mm
pSphA	135 / 549	73 / 318	5-8 mm
{ pNotB5	80 / 832	43 / 442	3-5 mm
{ pNotB7	21 / 302	-	3-5 mm
p5SpeCS	7 / 201	65 / 364	2-3 mm
pSmaSpe5.5	6 / 93	29 / 248	3-5 mm
pPvu8.5	4 / 342	3 / 138	2-3 mm (deformed)
pBam3.5	8 / 721	1 / 428	1 mm
pEco4.4	1 / 332	0 / 166	3-5 mm
p7XbaCS	0 / 302	0 / 79	
p5XbaCS	0 / 430	0 / 154	

B

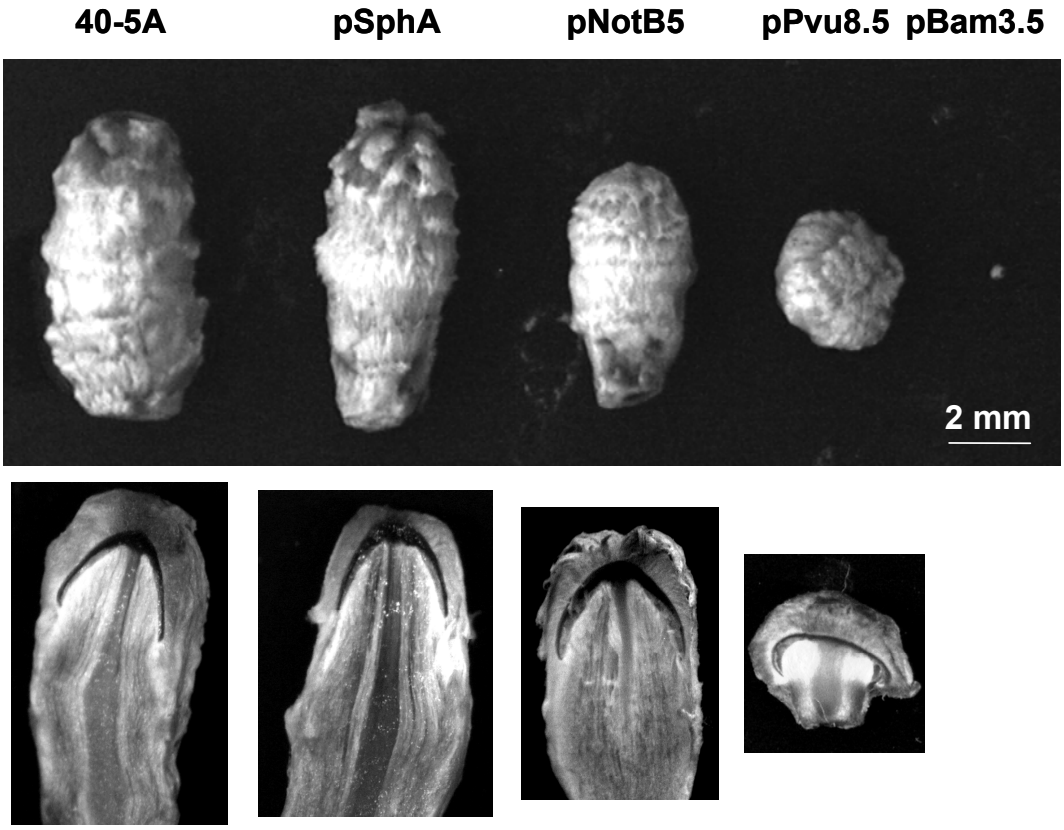


Figure 2. Identification of DNA fragments that restore fruiting body initiation in *C. cinerea* mutant 6-031. **A.** The three *C. cinerea* genomic *Not*I fragments (A, B, C) present in cosmid 40-5A are shown, as well as the length and position of subfragments present in pSphA. The *Not*I-B fragment and subfragments were inserted into either pBC SK(+) or pBluescript KS(-) (see Methods). For transformation we used either 1 μg of cosmid DNA or 1 μg DNA of pBC SK(+) or pBluescript KS(-) based plasmids plus 1 μg of pPAB1-2 for cotransformation (experimental setup I). To equalize the absolute number of DNA molecules possibly acting in fruiting body initiation, either 7 μg of cosmid 40-5A, 3.3 μg of pSphA or 1 μg/7 kb DNA of pBC SK(+) or pBluescript KS(-) based plasmids plus 1 μg of pPAB1-2 were applied (experimental setup II). Per single experiment, between 32 to 248 transformants were obtained. Since percentages of transformants initiating fruiting were comparable between different transformations of the same DNA construct (not shown), transformants of different experiments were added up. Transformation with 1 μg of pPAB1-2 and transformation with 1 μg of pPAB1-2 plus 1 μg of pBluecsript KS(-) or pBC SK(+) served as negative controls. From these control transformations, a total number of 1909, 215 and 132 transformants were respectively obtained and none of them initiated fruiting body formation. -, not performed. **B.** The morphological progress in primordia development declines when reducing the length of DNA fragments in transformation. The phenomenon is indicated by the isolated primordia formed by the cosmid 40-5A, pSphA, pNotB5, pPvu8.5 and pBam3.5 transformants (top panel). Differentiation of cap and stipe tissues is normal in primordia induced by cosmid 40-5A, pSphA and pNotB5. In contrast, the section through a primordium induced by plasmid pPvu8.5 shows that the internal pileus trama is missing (bottom panel).

4.4.4. The 3.5 kb *Bam*HI fragment is linked to the *skn1* mutation in strain 6-031

Strains 6-031 and JV6 have a distinct *Bgl*II restriction fragment length polymorphism (RFLP) in the DNA region covered by the 3.5 kb *Bam*HI fragment. 46 out of 588 randomly isolated descendants of a cross between the strains initiated fruiting body development on YMG/T medium. 41 of these clones had the RFLP pattern of monokaryon JV6. Both parental patterns were detected in the remaining 5 clones, but analysis of the *A* mating-type linked *pab1-1* allele (Srivilai et al., in preparation) in their oidia identified them as dikaryons (data not shown). Either the JV6 or the 6-031 RFLP pattern was found in 30 randomly isolated non-fruiting clones. The data suggest that the 3.5 kb *Bam*HI fragment is linked to the fruiting initiation defect (*skn1*) in mutant 6-031.

4.4.5. Transformation activities of cosmid 40-5A and derived subclones in *skn1*, *mat*$^+$, *bad*$^+$ strain OU3-1

To determine whether the quantitative and qualitative differences in complementation activity with different plasmids in mutant 6-031 were not triggered by the other mutations present in the strain, mutant strain OU3-1 carrying only the *skn1* mutation was selected from the *A43mut, B43mut* progeny of cross PS001-1 x 6-031. When transforming either cosmid 40-4A or its derivative pSphA into this strain (1 g DNA per transformation), transformation efficiencies of over 40% were achieved (61 positive/145 total cosmid 40-5A transformants; 56 positive/131 pSphA transformants). Mature fruiting bodies were obtained with this strain and these DNAs in 18 and 13% of the cases, respectively, (Fig. 3A), showing that the arrest in primordial development in cosmid 40-5A and pSphA transformants of mutant 6-031 was due to its *mat* mutation.

Transformation efficiencies with strain OU3-1 generally were higher than those with mutant 6-031 and quantitative differences in transformation efficiencies were less pronounced. Necertheless, in cotransformation of pBC SK(+) or a pBluescript (KS-) derivatives (usually 1 g) and pPAB1-2 (1 g), we once again observed quantitative differences in complementation efficiency. Unlike for the cosmids, no mature fruiting bodies were obtained using any of the plasmid constructs. pNotB7 initiated fruiting in strain OU3-1 in 20% of cases (24 positive/ 118 total transformants) but development arrested at a primordia size of ~5-6 mm. p5SpeCS, pSmaSpe5.5, pPvu8.5 and pBam3.5 were all less efficient in transformation with ~15% of transformants initiating fruiting (16 positive/110 pSmaSpe5.5 trnasformants, 24 positive/177 pPvu8.5 transformants, 29 positive/213 pBam3.5 transformants). Primordia development in the group of pSmaSpe5.5 transformants arrested at maximum sizes of 3-4 mm, in the group of pPvu8.5 transformants at a size of 2 mm. Most positive transformants of pBam3.5 had primodia of 1 mm in size, but eight transformants had primordial of 2-3 mm. Primordia from pPvu8.5 and pBam3.5

transformants were always malformed, being more flat than wild-type primordial. Moreover, they lacked inner tissues of the pileus (Figure 3, B and C). The results indicate again that pBam3.5 carries that gene for fruiting body initiation, although subsequent development does not follow the normal route. pBluescript KS (-) control transformants (51 clones) gave no positive transformants. Transformants of p5SpecCS (350 ng DNA were used) formed normal-shaped primordial with final sizes of 2-3 mm with a transformation of frequency of 6% (7 positive / 105 transformants).

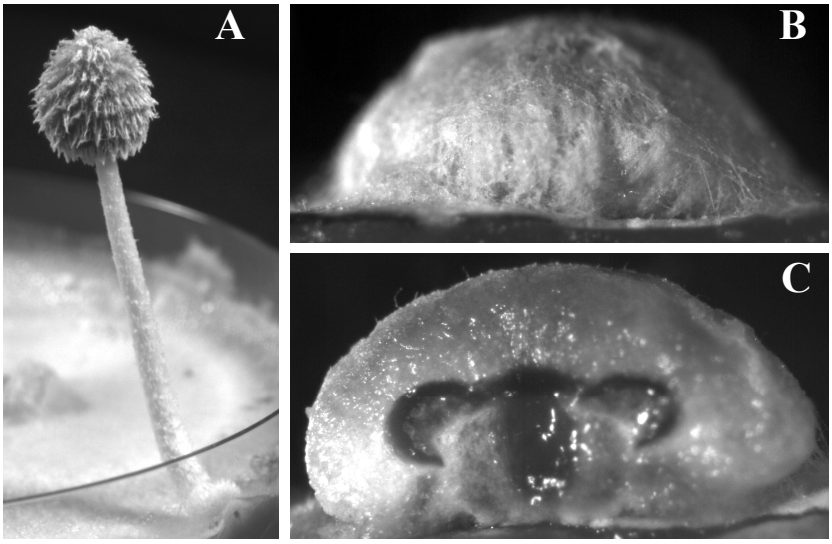


Figure 3. Fruiting behavior of transformants of homokaryon OU3-1. (A) Many transformants of cosmid 40-5A form mature fruiting bodies (~4 cm). (B and C) pBam3.5 transformants form small, flat primordia (2 mm primordium shown) that lack inner pileus tissue.

4.4.6. Characterization of the 10.5 kb *Not*I-B region

The quantitative and qualitative differences in complementation activities with different plasmids led us to sequence the whole genomic *Not*I-B region, which is 10526 bp in size. A GeneBank BLAST search with this sequence revealed four potential coding regions, whose deduced protein sequences showed highest similarities to the human ADP-ribosylation factor-like protein 2 ARL2 (67% identity and 82% similarity over a length of 185 aa; accession number P36404), the CFA synthase of *Escherichia coli* (32% identity and 48% similarity over a length of 367 aa; P30010), the galacturonosyl transferase Cap1E in *Streptococcus pneumoniae* (32% identity and 49% similarity over a length of 91 aa; L36873) and the C-terminal part of the kinesin-like protein UNC-104 in *Caenorhabditis elegans* (32% identity and 51% similarity over a length of 271 aa; P23678) (Fig. 4A). Whereas potential start codons are present on the *Not*I B-fragment fragment for the potential *ARL2*- and *cfa*-like genes, the *UNC-104*-related sequences is incomplete and 5´-truncated by the *Not*I site. For the stretch of DNA translating in a Cap1E-like sequence, there is no obvious startcodon. In accordance, a transcription analysis of the entire 10.5 kb *Not*I-B fragment

detected three transcripts (Fig. 4B), corresponding in location to the deduced coding regions for the ARL2-like protein (gene *arf1*), for the potential CFA (gene *cfs1*) and for the UNC-104 like kinesin (gene *kin1*). Weak transcripts for *arf1* were detected in Northern blots of total RNA. When using poly(A)$^+$ RNA to increase the sensitivity, transcripts for *arf1*, *cfs1* and *kin1* were well detected but we never observed a transcript for a further gene.

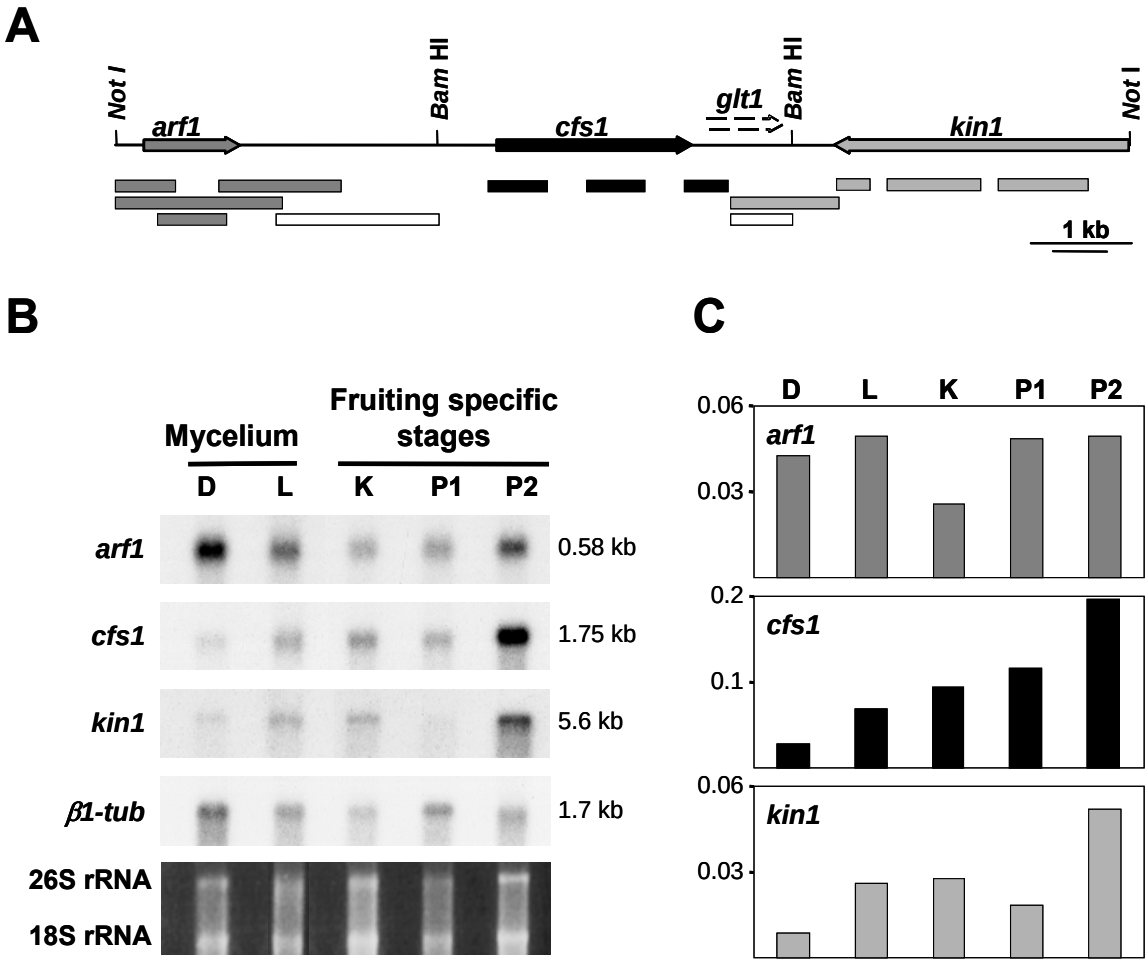


Figure 4. Transcription profile of the *Not*I-B region. **A.** Sequence analysis of pNotB5 revealed four potential *C. cinerea* open reading frames (indicated by the arrows *arf1*, *cfs1*, *glt1* and *kin1*), of which one (*kin1*) is only partially present on the *Not*I-B fragment and one (*glt1*) has no obvious startcodon. **B.** Three transcripts, *arf1*, *cfs1* and *kin1* were detected in Northern blot analysis by various DNA probes (shown as differentially shaded boxes in relation to the respective transcript detected). No signal was detected with two probes (open boxes) in the intergenic *arf1-cfs1* and *cfs1-kin1* regions. Gene expression was analyzed with poly(A)$^+$RNA isolated from vegetative mycelium grown on YMG/T agar at 37°C for 4 days in the dark (sample D) or 7 days in the light (sample L). Letter K denotes RNA isolated from cultures grown for 6 days at 37°C in the dark that developed numerous primary hyphal knots within the aerial mycelium. Letter P1 and P2 indicate RNA extracted from isolated 1mm sized primordia at a prekaryogamy stage and from isolated 1 cm sized primordia undergoing meiotic divisions, respectively. Primordia were harvested at day 3 (P1) and day 5 (P2) after fully grown YMG/T cultures were incubated at 25°C under standard fruiting conditions. 26S and 18S rRNA are shown to indicate the quality of poly(A)$^+$RNA samples. **C.** Densitometric quantification of the transcripts. For calibration, the β1-tubulin (*β1-tub*) transcript per poly(A)$^+$RNA was used as standard. Transcript levels are given in arbitrary units.

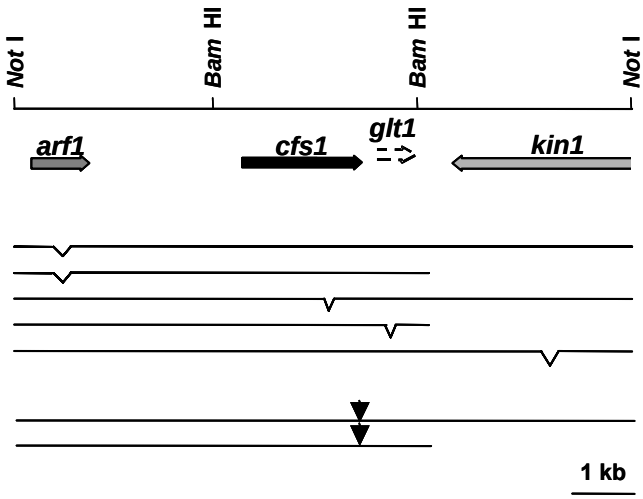
The *arf1* transcript is about 0.58 kb in size (Fig. 4B). *arf1* is expressed in all developmental stages tested (mycelia grown in constant dark and constant light, dark-grown mycelium containing primary hyphal knots, 1 mm-sized primordia at a prekaryogamy stage of tissue differentiation and 1 cm-sized primordia being after karyogamy in meiosis) at similar level, but decreases slightly at the stage of primary hyphal knot formation (Fig. 4C). The expression of the 1.75 kb sized *cfs1* transcript is poor in the dark (Fig. 4B). When a culture forms primary hyphal knots, light enhances *cfs1* transcription, which continues to increase from fruiting body initiation to primordial maturation (Fig. 4C). Gene *kin1* has a 5.6 kb transcript (Fig. 4B), which is poorly expressed in all stages except in primordia at the meiotic stage, just about to undergo rapid stipe elongation and cap expansion (Fig. 4C).

4.4.7. Gene *cfs1* is essential for fruiting body initiation and primordia maturation

The transformation data of mutants 6-031 (shown in Fig. 2) and OU3-1 did not yet allow to definitely assign the fruiting restoring ability of the 10.5 kb *Not*I-B fragment and the 3.5 kb *Bam*HI fragment to a single gene. The higher frequency of transformants initiating fruiting and the qualitative progress in primordia development with larger DNA fragments indicated that more than one of the cloned genes could contribute together or sequentially to fruiting body initiation. Southern blot analysis, using genomic DNA from mutant 6-031 and homokaryon AmutBmut digested with a number of restriction enzymes and three DNA probes that together covered the whole *Not*I-B fragment (a 4.4 kb *Not*I-*Kpn*I fragment, a 3.5 *Bam*HI fragment and a 3.5 kb *Spe*I-*Not*I fragment), excluded the possibility of a large deletion in this region in mutant 6-031 (not shown). Moreover, each probe detected only single bands (not shown), indicating the single copy nature of all cloned genes.

Next, we constructed plasmids with deletions within the coding region of *arf1*, *cfs* or *kin1* and within the uncertain *glt1* region (see Material and methods) and tested them by transforming mutant 6-031. Only plasmid p7NcoCSΔcfs, carrying a deletion in *cfs1*, lost the ability to restore fruiting body initiation in the mutant (Fig. 5), defining *cfs1* as the gene active in fruiting body initiation. Since interruptions within other genes did not fundamentally influence the transformation efficiency and since primordia formed were of normal shape and 2-4 mm in size as those obtained with the unmutated *Not*I-B fragment in same series of experiments (Fig. 5), it is possible that the general chromosomal environment of gene *cfs1* rather than the presence of other functional genes influences *cfs1* gene expression and accounts for the developmental differences observed in our transformation experiments.

In the *cfs1* allele from mutant 6-031, a single T to G transversion was found, which leads to a Y441D amino-acid substitution in the C-terminus of the deduced protein sequence (Fig. 6). When introducing this T to G transversion into the cloned *Not*I-B fragment and a smaller 7 kb *Not*I-*Spe*I fragment, neither of the two resulting plasmids pNotB5/6-031 and p5SpeCS/6-031 were able to restore fruiting body initiation (Fig. 5), indicating that the point mutation is indeed the cause of the inability to initiate fruiting body formation in mutant 6-031.



Construct	Positive/total transformants	Maximal primordium size
pNotB5Δ*arf*	12 / 293	2-3 mm
p5SpeCSΔ*arf*	5 / 218	2-3 mm
p7NcoCSΔ*cfs*	0 / 582	
p5SpeCSΔ*glt*	31 / 259	2-3 mm
p5BstCSΔ*kin*	15 / 260	3-4 mm
pNotB5/6-031	0 / 218	
p5SpeCS/6-031	0 / 302	

Figure 5. Gene *cfs1* is essential for fruiting body initiation. Plasmids pNotB5Δ*arf*, p5SpeCSΔ*arf* , p5SpeCSΔ*glt* and p5BctCSΔkin containing a deletion in either *arf1*, *kin1* or the *glt1* region (positions of deletions within subcloned fragments are indicated by open triangles) still permitted initiation of fruiting body formation when transformed into mutant 6-031. Positive transformants developed 2-4 mm primordia with normal morphology. In contrast, a deletion in *cfs1*$^{+}$ in plasmid p7NcoCSΔ*cfs* abolished the ability to induce fruiting body formation. Likewise, plasmids pNotB5/6-031 and p5SpeCS/6-031 carrying the T to G transversion found in the *cfs1* allele of mutant 6-031 (positions are marked by black arrow heads) did not activate fruiting body formation in mutant 6-031. In all transformations, 1 μg DNA/ 7 kb length of the test plasmids plus 1 μg of pPAB1-2 have been used.

4.4.8. The *cfs1* gene encodes a protein highly similar to bacterial cyclopropane fatty acid synthases

The cDNA of the *cfs1* gene is 1776 bp in size with a 66 bp long nucleotide sequence upstream of the first start codon ATG and a 300 bp long nucleotide sequence downstream of the stop codon TGA. Comparison of the genomic DNA and cDNA sequences revealed an open reading frame (ORF) of 1407 bp interrupted by 10 introns of 53 to 70 bp in size with typical *C. cinerea* 5´- and 3´-splice sites and branch-receptor sequences (Boulianne et al. 2000). The promoter region of *cfs1* contains a CAAT element 60-57 bp upstream of the transcription initiation site. No classical TATA box is found, but an AATAAAAA sequence is 37-30 bp upstream of the transcription

initiation site. 456 bp are upstream to the transcription initiation site in the 3.5 kb *Bam*HI fragment having the smallest sequence regularly but inefficiently active in transformation (Fig. 2). This sequence should mediate at least some promoter activity. Within the *cfs1* coding region, 332 bp downstream of the start codon ATG in the second exon is the *Eco*RI restriction site used to construct plasmid pEco4.4 and pEco4.4-pab with 5´ truncated *cfs1* copies. Homologous recombination at the natural *cfs1* locus within the *C. cinerea* genome might therefore explain the occasional transformants with well developed primordia obtained with these two plasmids (not shown).

The *cfs1* ORF encodes a polypeptide of 469 amino acid residues with a predicted molecular mass of 52 kDa. The Cfs1 protein has an overall high identity to a number of bacterial CFA synthases (Fig. 6), a particular subfamily of the S-adenosyl-L-methionine (SAM) dependent C-methyltransferases (MTases) (Fauman et al. 1999). NCBI database searches for related proteins also identified an enzyme from the plant *Sterculia foetida* (AF470622) and potential but not yet characterized eukaryotic protein products from *Arabidopsis thaliana* (BAB02771.1), *Oryza sativa* (AK0691115), from the worm *C. elegans* (T18571) and from the filamentous ascomycetes *Aspergillus nidulans* (EAA62781), *Neurospora crassa* (EAA32979) and *Magnaporthe grisea* (EAA50372) and *Ustilago maydis* (EAK81912) (Fig. 7). In contrast, no homologous human or any other mammal sequence seems to exist in the Genbank data bases. Within the fungi, no closely related sequences were found in the ascomycete yeasts *Eremothecium* (*Ashbya*) *gossypii*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. In the yeast *S. cerevisiae*, the most similar protein is ERG6, a Δ24-sterol-C-methyltransferase (31% identity and 48% similarity over a length of 236 aa; 19% identity and 33% similarity over the whole protein length; S42003; Fig. 6).

Consistent with a function as SAM-dependent methyltransferase, a SAM-binding motif is present in Cfs1 between aa 209 and 338 (Fig. 6). Protein profile programs (PSA and PSIpred) predict this region to adopt the typical 6 helices-7 strands configuration of SAM-binding domains (referred to as a SAM-dependent MTase fold) (Fauman et al. 1999). All but one amino acid shown to contact SAM in structurally characterized SAM-dependent MTases (Fauman et al. 1999) are conserved in Cfs1 (Fig. 6). Programs TMpred and TMHMM both predict potential transmembrane domains in the C-terminus of the *C. cinerea* Cfs1 protein (aa 435-453 and aa 437-456), closely behind the SAM-binding motif (Fig. 6). The Y441D amino acid substitution in mutant 6-031 is localized in this region (Fig. 6). This substitution overturns the prediction of a transmembrane domain and gives rise to higher hydrophilicity (Goldman/Engelman/Steitz prediction) at the C-terminal region (not shown). Overall, the wild-type Cfs1 protein appears to be hydrophilic without any long hydrophobic region.

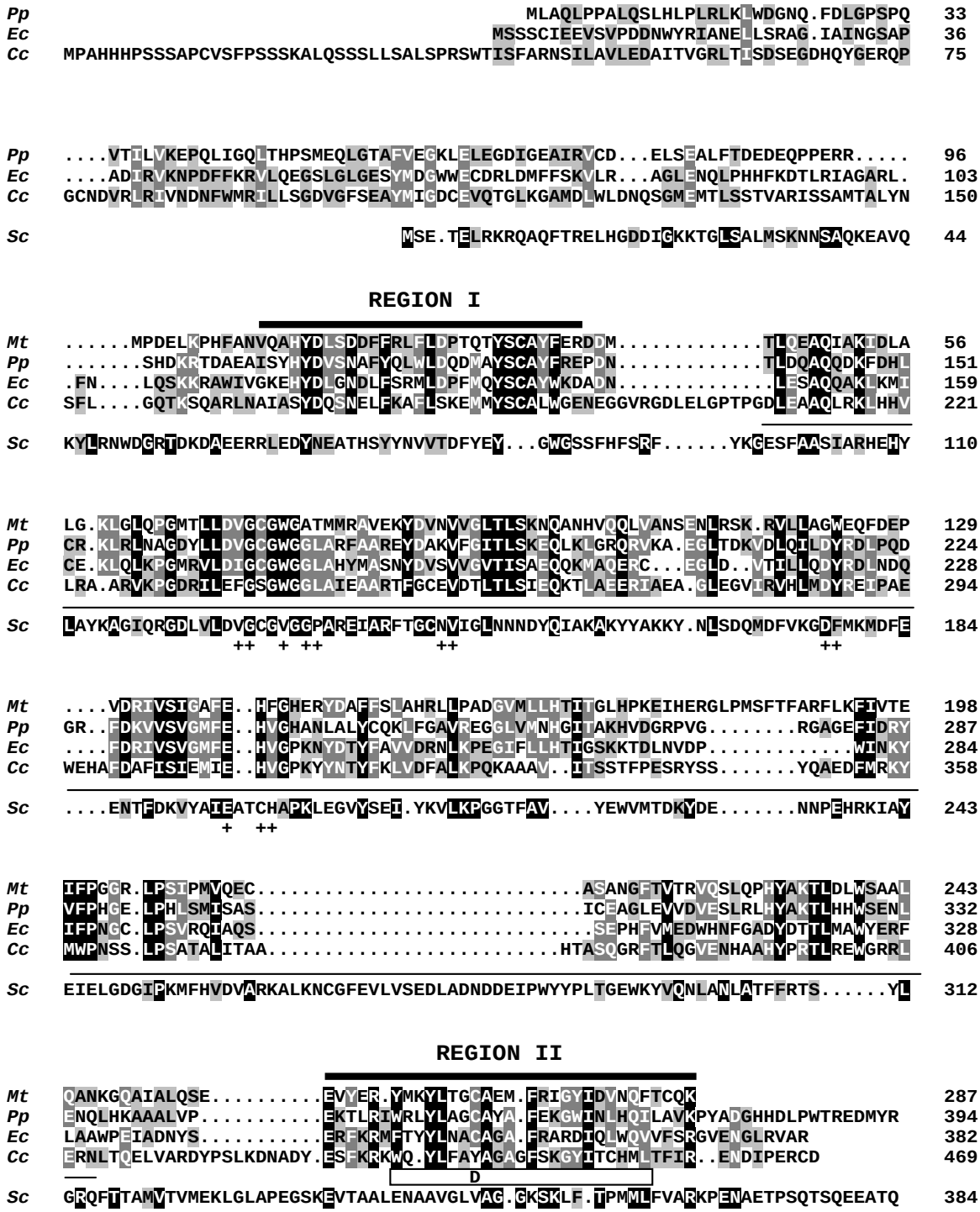


Figure 6. Sequence alignment of *C. cinerea* Cfs1 (*Cc*) to bacterial cyclopropane fatty acid synthases (*Mt*, *Pp*, *Ec*) and to yeast Δ24-sterol methyltransferase ERG6. *Pp*: protein ylp3 of the Gram$^-$ bacterium *Pseudomonas putida* (P31049); *Ec*: CFA of the Gram$^-$ bacterium *E. coli* (P30010); *Mt*: CFA1 of the Gram$^+$ bacterium *Mycobacterium tuberculosis* (Q11195); *Sc*: ERG6 of yeast *S. cerevisiae* (S42003). In the ERG6 protein sequence, amino acids identical to the *C. cinerea* Cfs1 sequence are shaded in black, similar ones in gray. A line marks the structurally conserved SAM-dependent MTase fold. A "+" indicates those amino acids shown in other proteins to contact SAM (Fauman *et al.*, 1999). The open box at the C-terminal ends of the proteins indicates potential membrane-interacting regions in Cfs1. The letter D indicates the amino acid exchange found in Cfs1 of mutant 6-031 at position Y441. Region I and region II denote protein regions specific to cyclopropane fatty acid synthase.

Supplementary Material on the Web: Figure. 7: Two conserved regions in potential CFA synthases. Region I in all (potential) bacterial and eukaryotic CFA synthases is directly N-terminal to the SAM-binding domain (compare Fig. 6 in this chapter). Region II is at the C-terminus of all proteins behind the SAM-binding domain. The consensus sequences of both regions (**Region I**: $V/I_{85}XXH_{88}\mathbf{Y_{100}D_{100}}V/L/I_{92}S_{77}\mathbf{N/D_{100}}D/N_{65}F_{65}\mathbf{F/Y_{100}}XL/I_{73}W/F_{85}L_{92}D_{77}P_{54}S/T_{69}M/L_{77}T/S_{58}\mathbf{Y_{100}S/T_{100}}C_{85}A_{92}Y/F_{54}\mathbf{F/W_{100}}E_{38}R/K_{38}$; **Region II**: $XV/M/L/I_{50}XXQ/E_{73}XXXR/K_{65}V/M/L/I_{54}\mathbf{Y/W/F_{100}}XXY_{96}L/M_{73}XXC_{69}A_{58}XX\mathbf{F_{100}}K/R_{58}XG_{58}XL/I/V_{81}D/N_{58}V/L_{73}XQ_{77}V/M/L/I_{62}T_{50}XK/R_{96}$) are given with the percentage of amino acid usage (numbers in subscript; in bold: positions with 100 % aa conservation). The boxed tyrosine (Y441) of the *C. cinerea* Cfs1 sequence (*Cc*) is substituted by an aspartic acid (D) in mutant 6-031. Proteins CFA2 (Q11196), umaA1 (CAA17424), CFA1 (Q11195), mmaA1 (B70614), mma2 (A70614), umaA2 (CAA17425), mmaA3 (AAC44618), Rv3720 (CAA18042) and ufaA1 (H70830) are from *M. tuberculosis* (strain H37RV). CmaA (AAC44876) is from *M. bovis* BCG and MLCB2407 (CAA19156) is from *M. leprae.* Other bacterial proteins are *Pp* from *Pseudomonas putida* (P31049), *Cj* from *Campylobacter jejuni* (CAB73437), *Hp* from *Helicobacter pylori* strain 26695 (H64571), *Aa* from *Aquifes aeolicus* (F70449), *Dr* from *Deinoccus radiodurans* strain R1 (AAF11731), *Sc* from *Streptomyces coelicolor* A3(2) (CAB89463), *Pa* from *P. aeruginosa* (AAG08931) and *Vc* from *Vibrio cholerae* (AAF94281). Eukaryotic proteins are *Ce* from *C. elegans* (T18571), *St* from *Sterculia foetida* (AAM33848), *At* from *A. thaliana* (BAB02771), *Dd* from *Dictostelium discoideum* (EAL65538) and *Nc* from *N. crassa* (EAA32979).

Supplementary Material on the Web: Figure. 7:

4.5. Discussion

In this study, we characterized the first mutant of the basidiomycete *C. cinerea* with a specific defect in fruiting body initiation, at the transition stage from primary to secondary hyphal knots. Through mutant complementation, we cloned gene *cfs1* that, by homology to heterologous genes, encodes a potential CFA synthase. Consistent with a function in fruiting body initiation, transcription of the *cfs1* is low in the dark, but induced by light. The gene is specifically active at the light dependent stage of secondary hyphal knot formation and in subsequent primordium development. When transforming the subcloned *cfs1* gene into the fruiting defective mutants 6-031 and OU3-1, we observed quantitative and qualitative differences in complementation activities with *cfs1*-carrying DNA fragments of different length, a phenomenon also observed in transformations with another gene acting early in fruiting body development in *Coprinopsis* (Clergeot et al., unpublished). Interruptions within the open reading frames of the neighboring genes (*arf1*, *kin1* and the '*glt1*' region present in the 10.5 kb *Not*I-B fragment) had no major effect on the complementation activity of *cfs1*, indicating that the likely cause for the differences in transformation activity is rather that the larger chromosomal environment plays a role in proper *cfs1* gene expression. Transcription of *arf1* is somewhat decreased at the onset of *cfs1* expression during primary hyphal knots formation, and *kin1* is specifically transcribed at subsequent stages of primordia formation when *cfs1* transcription is the highest. Possibly, the transcription profiles of *arf1* and *kin1* influence the expression of *cfs1* during early and later stages of fruiting body development.

4.5.1. Structure of the Cfs1 protein

Within the *C. cinerea* Cfs1 protein, we identified a potential SAM-binding domain, a "SAM-dependent methyltransferase (MTase) fold" (Fauman et al. 1999). CFA synthases are C-MTases that, in bacteria, transfer a methylene group from SAM to a C-atom in unsaturated membrane localized phospholipids thereby forming a cyclopropane ring (Grogan and Cronan 1997). Plant and fungal Δ^{24}-sterol C-MTase are the closest related to CFA synthases, possibly because their enzyme activities are both linked to lipid bilayers (Taylor and Cronan 1979; Leber et al. 1994). Low homology is found over the whole protein length between ERG6 of *S. cerevisiae* and Cfs1 of *C. cinerea*, with the highest conservation in the SAM-binding motif (Fig. 6). In ERG6, directly at the N-terminal end of the SAM-dependent MTase fold is a sequence (DFYEYGWGSSFHFS; residues 77-92) referred to as region I that is highly specific to all Δ^{24}-sterol C-MTases, has sterol binding activity and forms an -helix with a loop structure that targets into the substrate pocket (Nes et al. 1998, 1999, 2004). This sequence is not present in the family of CFA synthases (Fig. 6).

Instead, a highly specific sequence occupies the corresponding position (region I consensus: V/I_{85}XXH_{88}**Y**$_{100}$**D**$_{100}$V/L/$I_{92}$$S_{77}$**N**/**D**$_{100}$D/$N_{65}$$F_{65}$F/**Y**$_{100}$XL/$I_{73}$W/$F_{85}$$L_{92}$$D_{77}$$P_{54}$S/$T_{69}$M/$L_{77}$T/$S_{58}$**Y**$_{100}$**S**/ **T**$_{100}$$C_{85}$$A_{92}$Y/$F_{54}$**F**/**W**$_{100}$$E_{38}$R/$K_{38}$; see http://www.genetics.org/supplemental/ for the supplemental material). In the crystal structures of mycolic acid cyclopropane synthases from *Mycobacterium tuberculosis*, this CFA synthase signature sequence adopts a helix-loop-helix-loop structure that coat the surface of a hydrophobic tunnel from the entrance to active SAM binding site. The alkyl chains of model substrates enter these pockets in an U-shaped manner, thereby contacting amino acids from region I (Huang et al. 2002).

In *E. coli*, CFA synthase is a soluble protein found in the cell cytoplasm that uses SAM as a soluble and UFA (unsaturated fatty acids)-containing phospholipid as an insoluble substrate whilst accessing to both inner and outer leaflets of intact UFA-containing membranes (Taylor and Cronan 1979). The substrate C-double bond, positioned at 9-11 carbon unit from the glycerol backbone of the phospholipid molecule, is located deeply within the hydrophobic core of the membrane bilayer (Gally et al. 1979; Seelig and Seelig 1980). Inhibitor studies with sulfhydryl-modifying reagents and C-terminal truncation (50 aa) suggested that the C-terminus and possibly C354 being central to a domain now called region II (Fig. 6 and 7) have a role in catalysis or interaction with the membrane (Wang et al. 1992). However, changing C354 to an alanine or serine did not result in loss of function (Grogan and Cronan 1997; Courtois et al. 2004). The C-terminal region (region II IN Figure 6, consensus: XV/M/L/I_{50}XXQ/E_{37}XXXR/K_{65}V/M/L/I_{54}**Y**/**W**/**F**XXY_{96}L/M_{73}XXC_{69}A58XX **F**$_{100}$K/R_{58}XG_{58}XL/I/V_{81}D/N_{58}V/L_{73}XQ_{77}V/M/L/$I_{62}$$T_{50}$XK/$R_{96}$; see supplemental material at http://www.genetics.org/supplemental/) is nevertheless important, since the Y441D substitution in our mutant 6-031 resulted in a loss-of-function. Computer programs predict the wild-type Cfs1 of *C. cinerea* being a cytoplasmic enzyme, like the CFA synthase of *E. coli*, with two transmembrane domains in the C-terminus. Possibly, the C-terminus functions in anchoring the Cfs1 protein to the membrane and/or represents part of the catalytic domain. In the crystallized *M. tuberculosis* enzymes, the C-terminal end with region II forms an -helix and a -sheet. C269 at the C-terminus of this -helix (corresponding to C354 in *E. coli*) is in vicinity of the active site, whilst the -sheet dangles away from the site (Huang et al. 2002).

4.5.2. The role of cyclopropane fatty acid synthases and their products in bacteria

CFA synthases have been found in a broad range of bacteria, with *cis*-9,10-methylenehexadecanoic acid (17CFA), *cis*-9,10-methyleneoctadecanoic acid (MOA, DHSA = dihydrosterculic acid, C19) and *cis*-11,12-methyleneoctadecanoic acid (lactobacillic acid, C19) being characteristic bacterial CFAs (for review see Grogan and Cronan 1997). In *E. coli*, the CFA synthase is not essential for growth under an assortment of experimental conditions (Grogan and

Cronan 1986) but improves survival in low pH environment (Brown et al. 1997; Chang and Cronan 1999). In other bacteria, the production of CFAs also relates to stress conditions (for examples see Bodnaruk and Golden 1996; Valderrama et al. 1998; Broadbent and Lin 1999; Guillot et al. 2000; Mazumder et al. 2000; Kim et al. 2005). In consequence of CFA production, membrane properties, in particular membrane fluidity, alter with enhanced bacterial stress tolerance (Couto et al. 1996; Sajbidor 1997; Chang and Cronan 1999). Phospholipids containing CFAs have a broader transition temperature range and increased rigidity than those containing UFAs, which confers more resistance of the membrane lipid matrix to environmental perturbations (Dufourc et al. 1984; Perly et al. 1985). Furthermore, in the cell envelope of the pathogen *M. tuberculosis* cyclopropane mycolic acids of C60 to C90 are formed by a number of CFA synthases, shown to be necessary for cording, persistence and bacterial virulence (Glickman et al. 2000). The host innate immune activation is controlled by cyclopropane modification of glycolipid trehalose dimycolate in the bacterial cell envelope (Rao et al. 2005).

4.5.3. The role of cyclopropane fatty acid synthases and their products in eukaryotes

In eukaryotes, CFAs have only sporadically been reported, the chemical structures are far more diverse and origin and biological roles have still to be clarified. For example, cyclopropanated and brominated C18 straight-chain UFAs have been detected in lichens containing an ascomycete and either a green alga or a cyanobacterium (Rezanka and Dembitsky 1999). Cyclopropyl hydroxy-eicosanoids were described in a red alga (Nagle and Gerwick 1990), cyclopropanated C19 straight-chain fatty acid (cladocroic acid) in a sponge (D'Auria et al. 1993), cyclopropane containing eicosanoid (C20) in a soft coral (White and Jensen 1993) and 17CFA and DHSA in roe and in immature and mature individuals of the fish *Fundulus heteroclitus* whereby at least some of the CFAs might originate from the resident bacterial gut population (Cosper and Ackman 1983; Cosper et al. 1984). Feeding experiments in rats show that CFA from the diet can be specifically absorbed by intestinal tissues (Greter et al. 1979). The origin of 2,3-methylenehexadexadecanoic acid and 2,3-methyleneoctadecanoic acid from sheep rumen tissues (Body 1972), the 17CFA from submitochondrial particles of mammalian heart and liver tissues (Sakurada et al. 1999), the *cis*-3,4-methyleneoctadecanoic acid (a urinary metabolite of DHSA; Greter et al. 1979) and the *trans*- and *cis*-3-cyclopropane octanoylcarnitines (Libert et al. 1997) from human urine are not known.

DHSA has also been identified in trypanosomatid protozoa (Fish et al. 1981) and CFA synthase activity been demonstrated (Li et al. 1993). Eukaryotic CFA synthesis has now also been proven by heterologous expression of a cloned *Sterculia foetida* plant gene in tobacco cells leading to production of DHSA from oleoyl phospatidylcholine. In *S. foetida*, the gene is expressed in seeds (Bao et al. 2003). *cis*-9,10-methyleneheptadecanoic acid and DHSA occur in roots of *S. foetida* and

Ceiba pentandra (Kaimal and Lakshminarayana, 1970) and in seed oils of many plants, especially in immature seeds from Malvales (Yano et al. 1971; Fisher and Cherry 1983; Grondin et al. 1997). A long chain CFA containing 25 C-atoms was found in leaves of snow drop and cow parsley (Kuiper and Stuiver, 1972), CFAs with 17, 18 and 19 C-atoms in females and eggs, but not in males, of millipedes (Oudejans and van der Horst 1978) and connected to cold-hardiness and drought resistance in plants and desiccation tolerance in millipedes.

In vegetative cells of the slime mold *Polysphondylium pallidum*, the saturated CFAs content is relatively high, decreases substantially at the developmental transition from amoebae to aggregation-competent cells, while the unsaturated CFAs 17CFA and lactobacillic acid, ingested with *E. coli*, concomitantly increase (Saito and Ochiai 1998). PHYLPA, a lysophosphatidic acid composed of cyclic phosphate and cyclopropane-containing hexadecanoic acid from the slime mold *Physarum polycephalum*, selectively inhibits *in vitro* members of the DNA polymerase-α family and proliferation of human fibroblast cells through arresting cells at the G(1) or G(2) phase and hinders tumor invasion and metastasis, possibly all by eliciting an cellular increase in cAMP (Murakami-Murofushi et al. 1995; Mukai et al. 1999). In other studies, MOA directly activated protein kinase C and increased DNA synthesis in gastric epithelial cells and histamine- and dibutyryl cyclic AMP-stimulated acid secretion in parietal cells (Beil et al. 1998a,1998b), whereas 17CFA reduced the contractility of papillary muscles of guinea pigs and inhibited Mg^{2+}-ATPase activity (Sakurada et al. 2000). In *Dictyostelium discoideum*, excess of bacterial CFAs fail in cell-cell adhesion and fail to respond on a cAMP pulse (Matsuoka et al. 2004).

To our knowledge, no cyclopropanated moiety has so far been reported in higher fungal lipids. However, in the higher basidiomycetes, the unsaturated linoleic acid is the major constituent of fatty acids (Byrne and Brennan 1975; Solberg 1989; Song et al. 1989; Bonzom et al. 1999; Sakai and Kajiwara 2004). The related oleic acid is shown in *E. coli* to be a substrate for the action of CFA synthase (Marinari et al. 1974) and preliminary expression studies suggest that the *E. coli* and *C. cinerea* enzymes are at least partially interchangeable in function (Loos et al., unpublished).

In this study we conclude by analysis of mutant 6-031 that the *C. cinerea cfs1* gene is superfluous for vegetative mycelial growth, but essential for fruiting body development. It is possible that as in bacteria by the action of Cfs1 the physical properties of cellular membranes alter and that this is the trigger to initiate sexual morphogenesis in the fungus. Strikingly, in feeding experiments membrane interactive compounds such as sucrose esters of fatty acids, plant saponins and cerebrosides induced fruiting body development in various Basidiomycetes (Kawai 1989; Oita and Yanagi 1989; Mizushina et al. 1998; Magae et al. 2004; for review see Kües and Liu 2000). Membrane alteration has therefore been postulated to be a stress signal that promotes the fungus to shift from vegetative to reproductive growth (Oita and Yanagi 1989; Magae 1999).

4.6. Acknowledgements

We are very grateful to Ben Lu for help with nuclear staining. PS holds a PhD studentship from the Mahasarakham University, Thailand. Large parts of this work were financed by the Swiss National Science Foundation (grants 31-46940.96, 31-46940.96/2 and 31-59157.99) and the Eidgenössische Technische Hochschule, Zürich. The lab in Göttingen was funded by the DBU (Deutsche Bundesstiftung Umwelt).

4.7. References

Arima, T., M. Yamamoto, A. Hirata, S. Kawano, and T. Kamada, 2004 The *eln3* gene involved in fruiting body morphogenesis of *Coprinus cinereus* encodes a putative membrane protein with a general glycosyltransferase domain. Fungal Genet. Biol. **41:** 805-812.

Badalyan, S. M., E. Polak, R. Hermann, M. Aebi, and U. Kües, 2004 Role of peg formation in clamp cell fusion in homobasidiomycete fungi. J. Basic Microbiol. **44:** 167-177.

Bao, X., J. J. Thelen, G. Bonaventura, and J. B. Ohlrogge, 2003 Characterization of cyclopropane fatty-acid synthase from *Sterculia foetida*. J. Biol. Chem. **278:** 12846-12853.

Beil, W., C. Birkholz, S. Wagner, and K. F. Sewing, 1998a *Helicobacter pylori* fatty acid *cis* 9,10-methyleneoctadecanoic acid increases $Ca2^{+}$(i), activates protein kinase and stimulates acid secretion in parietal cells. Prostaglandins Leukot. Essent. Fatty Acids. **59:** 119-125.

Beil, W., B. Obst, S. Wagner, and K. F. Sewing, 1998b The *Helicobacter pylori* fatty acid *cis*-9,10-methyleneoctadecanoic acid stimulates protein kinase C and increases DNA synthesis of gastric HM02 cells. Br. J. Cancer. **77:** 1852-1856.

Bertossa, R. C., U. Kües, M. Aebi, and M. Künzler, 2004 Promoter analysis of *cgl2*, a galectin encoding gene transcribed during fruiting body formation in *Coprinopsis cinerea* (*Coprinus cinereus*). Fungal Genet. Biol. **41:** 1120-1131.

Bodnaruk, P. W., and D. A. Golden, 1996 Influence of pH and incubation temperature on fatty acid composition and virulence factors of *Yersinia enterocolitica*. Food Microbiol. **13:** 17-22.

Body, D. R., 1972 The occurrence of cyclopropane fatty acids in the phospholipids of sheep rumen tissues. FEBS Letters **27:** 5-8.

Bonzom, P. M. A., A. Nicolaou, M. Zloh, W. Baldeo, and W. A. Gibbons, 1999 NMR lipid profile of *Agaricus bisporus*. Phytochemistry **50:** 1311-1321.

Bottoli, A. P. F., K. Kertesz-Chaloupková, R. P. Boulianne, J. D. Granado, M. Aebi, and U. Kües, 1999 Rapid isolation of genes from an indexed genomic library of *C. cinereus* in a novel *pab1*$^{+}$ cosmid. J. Microbiol. Meth. **35:** 129-141.

Boulianne, R. P., Y. Liu, M. Aebi, B. C. Lu, and U. Kües, 2000 Fruiting body development in *Coprinus cinereus*: regulated expression of two galectins secreted by a non-classical pathway. Microbiology **146:** 1841-1853.

Broadbent, J. R., and C. Lin, 1999 Effect of heat shock or cold shock treatment on the resistance of *Lactococcus lactis* to freezing and lyophilization. Cryobiology **39:** 88-102.

Brown, J. L., T. Ross, T. A. McMeekin, and P. D. Nichols, 1997 Acid habituation of *Escherichia coli* and the potential role of cyclopropane fatty acids in low pH tolerance. Int. J. Food Microbiol. **37:** 163-173.

Byrne, P. F., and P. J. Brennan, 1975 The lipids of *Agaricus bisporus*. J Gen. Microbiol. **89:** 245-255.

Chang, Y. Y., and J. E. Cronan, Jr., 1999 Membrane cyclopropane fatty acid content is a major factor in acid resistance of *Escherichia coli*. Mol. Microbiol. **33:** 249-259.

Chomczynski, P., and N. Sacchi, 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:** 156-159.

Cosper, C. I., and R. G. Ackman, 1983 Occurrence of *cis*-9,10-methylenehexadecanoic and *cis*-9,10-methyleneoctadecanoic acids in the lipids of immature and mature *Fundulus heteroclitus* (L.) and in roe. Comp. Biochem. Physiol. B **75:** 649-654.

Cosper, C. I., L. C. Vining, and R. G. Ackman, 1984 Sources of cyclopropanoid fatty acids in the mummichog *Fundulus heteroclitus*. Marine Biol. **78:** 139-146.

Courtois, F., C. Guérard, X. Thomas, and O. Ploux, 2004 *Escherichia coli* cyclopropane fatty acid synthase. Mechanistic and site-directed mutagenic studies. Eur. J. Biochem. **271:** 4769-4778.

Couto, J. A., N. Rozes, and T. Hogg, 1996 Ethanol-induced changes in the fatty acid composition of *Lactobacillus hilgardii*, its effects on plasma membrane fluidity and relationship with ethanol tolerance. J. App. Bacteriol. **81:** 126-132.

D'Auria, M. V., L. G. Paloma, L. Minale, R. Riccio, and A. Zampella, 1993 Metabolites of the New Caledonian sponge *Cladocroce incurvata*. J. Nat. Prod. **56:** 418-423.

Dufourc, E. J., I. C. Smith, and H. C. Jarrell, 1984 Role of cyclopropane moieties in the lipid properties of biological membranes: A ^{2}H NMR structural and dynamical approach. Biochemistry **23:** 2300-2309.

Fauman, E. B., R. M. Blumenthal, and X. D. Cheng, 1999 Structure and evolution of AdoMet-dependent methyltransferases, pp. 1-38 in *S-Adenosylmethionine-Dependent Methyltransferases: Structures and Functions*, edited by X. D. Cheng and R. M. Blumenthal. World Scientific, Singapore.

Fish, W. R., G. G. Holz, Jr., D. H. Beach, E. Owen, and G. E. Anekwe, 1981 The cyclopropane fatty acid of trypanosomatids. Mol. Biochem. Parasitol. **3:** 103-115.

Fisher, G. S., and J. P. Cherry, 1983 Variation of cyclopropenoid fatty acids in cottonseed lipids. Lipids **18:** 589-594.

Gally, H. U., G. Pluschke, P. Overath, and J. Seelig, 1979 Structure of *Escherichia coli* membranes. Phospholipid conformation in model membranes and cells as studied by deuterium magnetic resonance. Biochemistry **18:** 5605-5610.

Glickman, M. S., J. S. Cox, and W. R. Jacobs, Jr., 2000 A novel mycolic acid cyclopropane synthetase is required for cording, persistence, and virulence of *Mycobacterium tuberculosis*. Mol. Cell. **5:** 717-727.

Granado, J. D., K. Kertesz-Chaloupková, M. Aebi, and U. Kües, 1997 Restriction enzyme-mediated DNA intergration in *Coprinus cinereus*. Mol. Gen. Genet. **256:** 28-36.

Greter, J., S. Lindstedt, and G. Steen, 1979 Urinary metabolites of *cis*-9,10-methylene octadecanoic acid. *cis*-3,4-Methylene hexanedioic acid and related compounds. J. Biol. Chem. **254:** 2807-2813.

Grogan, D. W., and J. E. Cronan, Jr., 1986 Characterization of *Escherichia coli* mutants completely defective in synthesis of cyclopropane fatty acids. J. Bacteriol. **166:** 872-877.

Grogan, D. W., and J. E. Cronan, Jr., 1997 Cyclopropane ring formation in membrane lipids of bacteria. Microbiol. Mol. Biol. Rev. **61:** 429-441.

Grondin, I., J. Smadja, M. Farines, and J. Soulier, 1997 Les triacylglycérols de deux huiles de sapindacées: études des lipides de *Litchi sinensis* Sonn. et *Euphoria longana* Lam. Fondamental **4:** 295-300.

Guillot, A., D. Obis, and M. Y. Mistou, 2000 Fatty acid membrane composition and activation of glycine-betaine transport in *Lactococcus lactis* subjected to osmotic stress. Int. J. Food Microbiol. **55:** 47-51.

Huang, C., C. V. Smith, M. S. Glickman, W. R. Jacobs, Jr., and J. C. Sacchettini, 2002 Crystal structure of myolic acid cyclopropane synthase from *Mycobacterium tubercolosis*. J. Biol. Chem. **277:** 11559-11569.

Kaimal, T. N. B., and G. Lakshminarayana, 1970 Fatty acid compositions of lipids isolated from different parts of *Ceiba pentandra, Sterculia foetida* and *Hydnocarpus wightiana*. Phytochem. **9:** 2225-2229.

Kawai, G., 1989 Molecular species of cerebrosides in fruiting bodies of *Lentinus edodes* and their biological activity. Biochim. Biophys. Acta. **1001:** 185-190.

Kertesz-Chaloupková, K., P. J. Walser, J. D. Granado, M. Aebi, and U. Kües, 1998 Blue light overrides repression of asexual sporulation by mating type genes in the basidiomycete *Coprinus cinereus*. Fungal Genet. Biol. **23:** 95-109.

Kim, B. H., S. Kim, H. G. Kim, J. Lee, I. S. Lee *et al.*, 2005 The formation of cyclopropane fatty acids in *Salmonella enterica* serovar Typhimurium. Microbiology **151:** 209-218.

Kües, U., 2000 Life history and developmental processes in the basidiomycete *Coprinus cinereus*. Microbiol. Mol. Biol. Rev. **64:** 316-53.

Kües, U., and Y. Liu, 2000 Fruiting body production in basidiomycetes. Appl. Microbiol. Biotechnol. **54:** 141-152.

Kües, U., J. D. Granado, R. Hermann, R. P. Boulianne, K. Kertesz-Chaloupková, *et al.*, 1998 The *A* mating type and blue light regulate all known differentiation processes in the basidiomycete *Coprinus cinereus*. Mol. Gen. Genet. **260:** 81-91.

Kües, U., E. Polak, A. P. F. Bottoli, M. Hollenstein, P. J. Walser *et al.*, 2002a Vegetative development in *Coprinus cinereus*, pp. 133-164 in *Molecular Biology of Fungal Development*, edited by H. D. Osiewacz. Marcel Dekker, New York.

Kües, U., P. J. Walser, M. J. Klaus, and M. Aebi, 2002b Influence of activated *A* and *B* mating type pathways on developmental processes in the basidiomycete *Coprinus cinereus*. Mol. Genet. Genom. **268:** 262-271.

Kües, U., Künzler, M., Bottoli, A. P. F., Walser, P. J., Granado, J. D. *et al.*, 2004 Mushroom development in higher basidiomycetes; implications for human and animal health, pp. 431-470 in *Fungi in Human and Animal Health*, edited by R. K. S. Kushwaha. Scientific Publishers, Jodhpur, India.

Kuiper, P. J. C., and B. Stuiver, 1972 Cyclopropane fatty acids in relation to earliness in spring and drought tolerance in plants. Plant Physiol. **49:** 307-309.

Leber, R., E. Zinser, G. Zellnig, F. Paltauf, and G. Daum, 1994 Characterization of lipid particles of the yeast, *Saccharomyces cerevisiae*. Yeast **10:** 1421-1428.

Li, R. X., S. Ganguli, and R. A. Pascal, Jr., 1993 Synthesis of sulfur-substituted phosphatidylethanolamines and inhibition of protozoan cyclopropane fatty acid synthase. Tetrahedron Letters **34:** 1279-1282.

Libert, R., F. Van Hoof, M. Thillaye, M. F. Vincent, M. C. Nassogne *et al.*, 1997 Identification of new medium-chain acylcarnitines present in normal human urine. Anal. Biochem. **251:** 196-205.

Lu, B. C., and N.B. Raju, 1970 Meiosis in *Coprinus.* II. Chromosome pairing and the lampbrush diplotene stage of meiotic prophase. Chromosoma **29:** 305-316.

Lu, B. C., N. Gallo, and U. Kües, 2003 White-cap mutants and meiotic apoptosis in the basidiomycete *Coprinus cinereus*. Fungal Genet. Biol. **39:** 82-93.

Magae, Y., 1999 Saponin stimulates fruiting of the edible basidiomycete *Pleurotus ostreatus*. Biosci. Biotechnol. Biochem. **63:** 1840-1842.

Magae, Y., T. Nishimura, and S. Ohara, 2004 3-O-alkyl-D-glucose derivatives induce fruit bodies of *Pleurotus*. Mycol. Res. **109:** 374-376.

Marchuk, D., M. Drumm, A. Saulino, and F. S. Collins, 1991 Construction of T-vectors, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Res. **19**: 1154.

Marinari, L. A., H. Goldfine, and C. Panos, 1974 Specificity of cyclopropane fatty acid synthesis in *Escherichia coli*. Utilization of isomers of monounsaturated fatty acid. Biochemistry **13:** 1978-1983.

Matsuoka, S., H. Kuwayama, D. Ikeno, M. Oyama, and M. Maeda, 2004 Defect in peroximal multifunctional enzyme MFE1 affects cAMP relay in *Dictyostelium*. Dev. Growth Differ. **46:** 195-199.

Mazumder R., H. C. Pinkart, P. S. Alban, T. J. Phelps, and R. E. Benoit, 2000 Low-substrate regulated microaerophilic behavior as a stress response of aquatic and soil bacteria. Curr, Microbiol **41:** 79-83.

Mizushina, Y., L. Hanashima, T. Yamaguchi, M. Takemura, F. Sugawara *et al.*, 1998 A mushroom fruiting body-inducing substance inhibits activities of replicative DNA polymerases. Biochem. Biophys. Res. Commun. **249:** 17-22.

Moore, D., M. M. Y. Elhiti, and R. D. Butler, 1979 Morphogenesis of the carpophore of *Coprinus cinereus*. New Phytol. **83:** 695-722.

Mukai, M., F. Imamura, M. Ayaki, K. Shinkai, T. Iwasaki *et al.*, 1999 Inhibition of tumor invasion and metastasis by a novel lysophosphatidic acid (cyclic LPA). Int. J. Cancer **81:** 918-922.

Muraguchi, H., and T. Kamada, 1998 The *ich1* gene of the mushroom *Coprinus cinereus* is essential for pileus formation in fruiting. Development **125:** 3133-3141.

Muraguchi, H., and T. Kamada, 2000 A mutation in the *eln2* gene encoding a cytochrome P450 of *Coprinus cinereus* affects mushroom morphogenesis. Fungal Genet. Biol. **29:** 49-59.

Murakami-Murofushi, K., S. Kobayashi, K. Onimura, M. Matsumoto, M. Shioda *et al.*, 1995 Selective inhibition of DNA polymerase-alpha family with chemically synthesized derivatives of PHYLPA, a unique *Physarum* lysophosphatidic acid. Biochim. Biophys. Acta **1258:** 57-60.

Murata, Y., M. Fujii, M. E. Zolan, and T. Kamada, 1998 Molecular analysis of *pcc*1, a gene that leads to *A*-regulated sexual morphogenesis in *Coprinus cinereus*. Genetics **149:** 1753-1761.

Nagle, D. G., and W. H. Gerwick, 1990 Isolation and structure of constanolactones A and B, new cyclopropyl hydroxyeicosanoids from the temperate red alga *Constantinea simplex*. Tetrahedron Letters **31:** 2995-2998.

Nes, W. D., B. S. McCourt, W. X. Zhou, J. Ma, J. A. Marshall *et al.*, 1998 Overexpression, purification, and stereochemical studies of the recombinant (S)-adenosyl-L-methionine: delta 24(25)- to delta 24(28)-sterol methyl transferase enzyme from *Saccharomyces cerevisiae*. Arch. Biochem. Biophys. **353:** 297-311.

Nes, W. D., B. S. McCourt, J. A. Marshall, J. Ma, A. L. Dennis *et al.*, 1999 Site-directed mutagenesis of the sterol methyl transferase active site from *Saccharomyces cerevisiae* results in formation of novel 24-ethyl sterols. J. Org. Chem. **64:** 1535-1542.

Nes, W. D., P. Jayasimha, W. Zhou, R. Kanagasabai, C. Jin *et al.*, 2004 Sterol methyltransferase: functional analysis of highly conserved residues by site-directed mutagenesis. Biochemistry **43:** 569-576.

Oita, S., and S. O. Yanagi, 1993 Stimulation of *Schizophyllum commune* fruit body formation by inhibitor of membrane function and cell wall synthesis. Biosci. Biotech. Biochem. **57:** 1270-1274.

Oudejans, R. C. H. M., and D. J. van der Horst, 1978 Cyclopropane fatty acids in millipedes: their occurrence and metabolism. Abh. Naturwiss. Ver. Hamburg **21/22:** 345-348.

Perly, B., I. C. Smith, and H. C. Jarrell, 1985 Effects of replacement of a double bond by a cyclopropane ring in phosphatidylethanolamines: a ^{2}H NMR study of phase transitions and molecular organization. Biochemistry **24:** 1055-1063.

Polak, E., 1999 *Asexual Sporulation in the Basidiomycete Coprinus cinereus*. Ph.D. thesis, ETH Zurich, Zurich, Switzerland.

Rao, V., N. Fujiwara, S. A. Porcelli, and M. S. Glickman, 2005 *Mycopbacterium tuberculosis* controls host innate immune activation through cyclopropane modification of a glycolid effector molecule. J. Exp. Med. **201:** 535-543.

Rezanka, T., and V. Dembitsky, 1999 Novel brominated lipidic compounds from lichens of central Asia Phytochemistry **51:** 963-968.

Saito, T., and H. Ochiai, 1998 Fatty acid composition of the cellular slime mold *Polysphondylium pallidum*. Lipids **33:** 327-332.

Sajbidor, J., 1997 Effect of some environmental factors on the content and composition of microbial membrane lipids. Crit. Rev. Biotechnol. **17:** 87-103.

Sakai, H, and S. Kajiwara, 2004 Membrane lipid profile of an edible basidiomycete *Lentinula edodes* during growth and cell differentiation. Lipids **39:** 67-73.

Sakurada, K., H. Iwase, T. Takatori, M. Nagao, M. Nakajima, *et al.*, 1999 Identification of *cis*-9,10-methylenehexadecanoic acid in submitochondrial particles of bovine heart. Biochim. Biophys. Acta **25:** 214-222.

Sakurada, K., H. Iwase, M. Kobayashi, H. Uemura, H. Nakaya *et al*., 2000 cis-9,10-methylenehexadecanoic acid inhibits contractility and actomyosin ATPase activity of guinea pig myocardium. Biochem. Biophys. Res. Com. **274:** 533-536.

Sambrook, J., E. F. Fritsch, and T. Maniatis, 1989 *Molecular cloning: A laboratory manual*, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Seelig, J., and A. Seelig, 1980 Lipid conformation in model membranes and biological membranes. Q. Rev. Biophys. **13:** 19-61.

Solberg, Y., 1989 A literature review of the lipid constituents of higher fungi, new investigation of *Agaricus* species. Int. J. Mycol. Lichenol. **4:** 137-154.

Song, C. H., K. Y. Cho, N. G. Nair, and J. Vine, 1989 Growth stimulation and lipid synthesis in *Lentinus edodes*. Mycologia **81:** 514-522.

Swamy, S., I. Uno, and T. Ishikawa, 1984 Morphogenetic effects of mutations at the *A* and *B* incompatibility factors in *Coprinus cinereus*. J. Gen. Microbiol. **130:** 3219-3224.

Taylor, F. R., and J. E., Cronan, Jr., 1979 Cyclopropane fatty acid synthase of *Escherichia coli*: stabilization, purification, and interaction with phospholipid vesicles. Biochemistry **18:** 3292-3300.

Valderrama, M. J., M. Monteoliva-Sanchez, E. Quesada, and A. Ramos-Cormenzana, 1998 Influence of salt concentration on the cellular fatty acid composition of the moderately halophilic bacterium *Halomonas salina*. Res. Microbiol., **149:** 675-679.

Walser, P. J., R. Velagapudi, M. Aebi, and U. Kües, 2003 Extracellular matrix proteins in mushroom development. Recent Res. Devel. Microbiol. **7:** 381-415.

Wang, A. Y., D. W. Grogan, and J. E. Cronan, Jr., 1992 Cyclopropane fatty acid synthase of *Escherichia coli*: deduced amino acid sequence, purification, and studies of the enzyme active site. Biochemistry **31:** 11020-11028.

White, J. D., and M. S. Jensen, 1993 Biomimetic synthesis of a cyclopropane containing eicosanoid from the coral *Plexaura homomalla* Assignment of relative configuration. J. Am. Chem. Soc. **115:** 2970-2971.

Yano, I., B. W. Nichols, L. J. Morris, and A. T. James, 1971 The distribution of cyclopropane and cyclopropene fatty acids in higher plants (Malvaceae). Lipids **7:** 30-34.

Zolan, M. E., and P. J. Pukkila, 1986 Inheritance of DNA methylation in *Coprinus cinereus*. Mol. Cell. Biol. **6:** 195-200.

CHAPTER 5

Heterologous expression of mating type genes in basidiomycetes

Srivilai P., James T. Y., Vilgalys R., Chaisaena W., and Kües U.

Published in: VI Genetic and Cellular Biology of Basidiomycetes (G. Pisabarro and L. Ramírez, eds.), Universidad Pública de Navarra, Pamplona, Spain, *in press*.

This manuscript introduces shortly the background on the mating type genes in higher basidiomycetes and presents transformation into monokaryons an easy method to test mating type genes in heterologous genetic backgrounds. Examples of successful heterologous expression of genes from the same species as well as from the related species *Coprinopsis scobicola* (formerly *Coprinus bilanatus*) are given from the literature. Experiments shown in Figure 3 and Figure 5 were performed by Prof. Dr. Ursel Kües, others in monokaryon 218 and in the *A*null strain NA2 by myself. Other authors contributed discussion points as a basis of common future research projects.

5.1. Abstract

Mating type genes in basidiomycetes encode two types of transcription factors (HD1 and HD2) and pheromones and pheromone receptors. Usually, mating type genes are so dissimilar in DNA sequence (allelic genes and genes from different species) that they do not cross-hybridize. In homobasidiomycetes, directly next to the *A* mating type locus encoding the transcription factors is a highly conserved gene *mip* that allows positional cloning. A candidate gene for positional cloning of the *B* mating type genes encoding the pheromone-pheromone receptor system is *cla4*/*ste20*. With more and more mating type loci cloned from different species, evolution of these loci and their genes can be addressed by sequence analysis and by function by transformation into other species, here *Coprinopsis cinerea*. Transformation of cloned mating type genes into heterologous hosts can lead to activation of mating type controlled development. Heterologous expression of mating type genes is especially interesting for species in which no transformation system exists. Since in *C. cinerea* an *A* null-mutant is available without functional transcription factor genes, self-compatibility of cloned *A* genes from homothallic species can also be tested.

5.2. Introduction

5.2.1. Breeding systems in the basidiomycetes

An estimated 85-90% of all basidiomycetous species are heterothallic and need to undergo mating between two compatible monokaryotic strains for sexual development (karyogamy and meiosis) to occur. The remaining 10-15% of species are homothallic, i.e. self-compatible. These fertile homokaryotic strains undergo karyogamy and meiosis without mating to another strain. From such truly homothallic species, secondarily homothallic species have to be distinguished. Secondarily homothallic species carry two nuclei of opposite mating type in the basidiospores which germinate into fertile dikaryons (Whitehouse 1949; Quintanilha and Pinto-Lopes 1950).

Sexual development in heterothallic species is controlled by either one or two mating type loci. Species with one mating type locus are called bipolar, because two different mating type specificities segregate in the haploid progeny of a cross. These mating types correspond to the mating types of the parental strains of the cross. Species with two mating type loci are tetrapolar, and four different mating types are found among the progeny of a cross. Two of the four mating types are parental by passing on parental alleles at both mating type loci. The two alternate mating types are newly formed by recombination between the two mating type loci. Approximately 30-40% of heterothallic species are estimated to be bipolar and 60-70% to be tetrapolar (Whitehouse 1949; Quintanilha and Pinto-Lopes 1950).

In most cases, the single mating type locus of bipolar species are called *A*, the two mating type loci of tetrapolar species *A* and *B*. Usually, mating type loci in the basidiomycetes are multi-allelic. Different mating type specificities are indicated by numbers (*A1*, *A2*, *A3*, *B1*, *B2*, *B3*). In species with two mating type loci, every distinct *A* and *B* combination defines a specific mating type, i.e. *A1B1*, *A1B2*, *A2B1* and *A2B2* strains are all different in mating behaviour. Of these, *A1B1* and *A2B2* strains are compatible as are *A1B2* and *A2B1* strains. For a successful mating, fusing monokaryons need to be different at both mating type loci. In consequence, tetrapolar breeding systems promote outbreeding (Whitehouse 1949; Quintanilha and Pinto-Lopes 1950).

The *A* and the *B* mating type genes in tetrapolar species regulate different steps in sexual development explaining why both loci have to be different between two mates (for details see reviews by Casselton and Olesnicky 1998; Kües 2000; Kües et al. 2002a, 2004; Casselton and Riquelme 2005).

5.2.2. The mating type loci in tetrapolar species

Mating type loci have been cloned and functionally analyzed from the tetrapolar species *Coprinopsis cinerea*, *Schizophyllum commune* and *Ustilago maydis*. The *A* mating type loci of *C. cinerea* and *S. commune* and the *b* mating type locus of *U. maydis* contain genes encoding two types of homeodomain transcription factors known as HD1 and HD2 proteins. Generally, to induce sexual development, an HD1 protein from one mate has to interact with an HD2 protein of the other mate (Fig. 1). In the simple case in *U. maydis*, there is one pair of divergently transcribed *HD1* and *HD2* genes (*bE* and *bW*) with about 25 different alleles. In a mating reaction, there are therefore two compatible genes for proteins of the different classes of homeodomain transcription factors (HD1 and HD2) are specified by the first number. The second number defines the allele of a respective gene. DNA dissimilarities between alleles are indicated by boxes of different shading. The more conserved regions encoding the homeodomain DNA-binding motif are given in black boxes. The two possible functional interactions between products from allelic gene pairs are indicated by the arrowheads. These HD1-1/HD2-2 and HD1-2/HD2-1 interactions are redundant in function, protein interactions: bE1-bW2 and bE2-bW1 (compare Fig. 1).

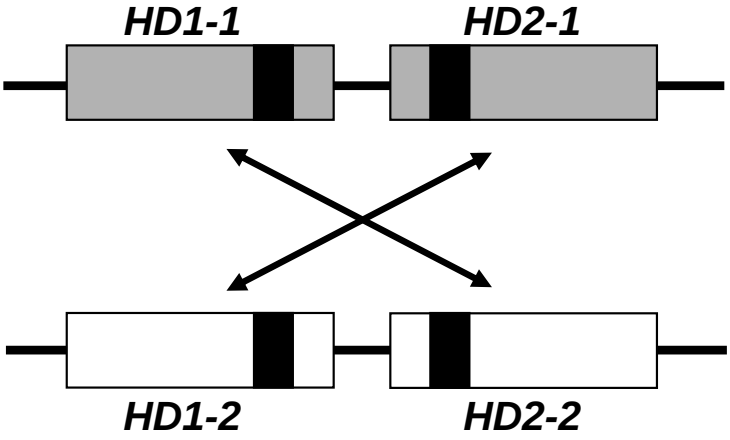


Figure 1. Schematic presentation of a mating type locus encoding *HD1* and *HD2* genes for homeodomain transcription factors. The simplest case with one pair of divergently transcribed genes are shown.

However, one such protein combination is sufficient. In *C. cinerea*, there are at least three independently interacting *HD1-HD2* gene pairs. Each gene pair has a few alleles that are freely recombining with the alleles from the other gene pairs. This recombination generates the estimated number of 160 different *A* mating type specificities. For sexual development, it is, however, enough

if mates carry different alleles at any one of the three possible *HD1-HD2* gene pairs. In *S. commune* with a high number of different *A* mating type specificities (>280), the situation is similar. So far, only one complete gene pair (*Aα* locus) has been characterized in this species and one non-allelic *HD2* gene (from the *Aβ* locus) been cloned (for details see reviews by Hiscock and Kües 1999; Casselton and Olesnicki 1998; Casselton and Challen 2005).

The *a* mating type locus of *U. maydis* and the *B* mating type loci of *C. cinerea* and *S. commune* contain genes for pheromones and pheromone receptors (Fig. 2). Pheromones and pheromone receptors of different specificity have to interact for induction of sexual development. In *U. maydis*, there are only two alleles of the *a* locus and each has a pheromone gene and a receptor gene. Three independent groups of genes, each comprising one pheromone receptor gene and up to three pheromone genes, have been described in *C. cinerea*. Different combinations of the various alleles of these groups are expected to give rise to the 80 *B* specificities estimated to exist in nature. Two groups with each one pheromone receptor gene and up to 8 different pheromone genes are described for the *B* locus in *S. commune*. Similarly for this species, 80 different *B* specificities are expected worldwide to exist (for details see reviews by Casselton and Olesnicky 1998; Kothe 2001; Casselton and Challen 2005; Riquelme et al. 2005).

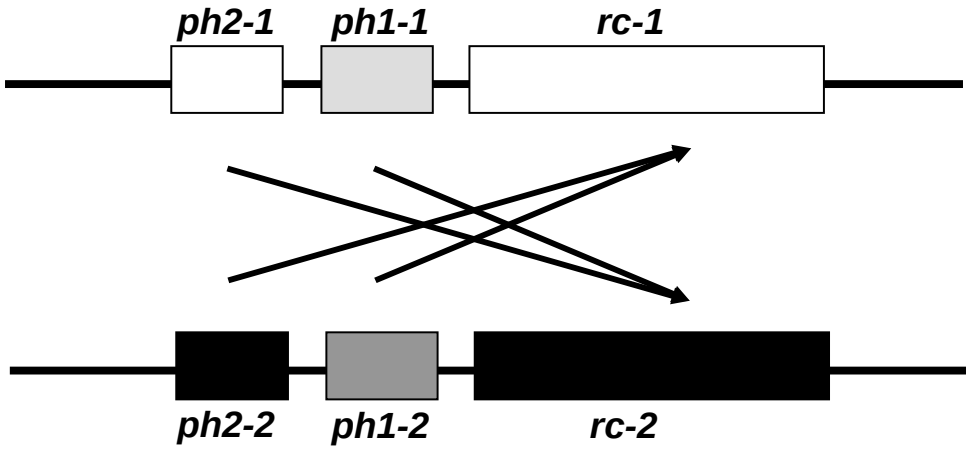


Figure 2. A hypothetical mating type locus encoding two different pheromones (Ph1, Ph2; second number = allele) and one pheromone receptor (Rc; second number = allele) is shown – in reality. There might be less or more pheromone genes and also genes unlinked to mating function that have been integrated into the locus by coincidence. Interactions of pheromones with the respective receptor from an allelic mating type locus are indicated by arrows.

5.2.3. Cloning mating type loci

Alleles of mating type loci are very dissimilar in sequence so that they do not cross-hybridize. This feature has been used in cloning the *B* mating type genes from *C. cinerea* by a genomic subtraction technique selecting unique DNA sequences for incorporation into a cloning vector (O´Shea et al. 1998). In other cases, as in cloning the *B* genes from *S. commune*, mating type genes were identified upon transformation through activation of developmental programs being under control of the mating type genes (Specht 1995). Chromosome walking from closely linked and easily to identify metabolic genes was applied in cloning *A* genes from both *C. cinerea* and *S. commune* (Giasson et al. 1989; Mutasa et al. 1990). Despite the reported homothallic behaviour of strains in *Phanerochaete chrysosporium* (Alic et al. 1987), mating type genes were identified in the genome sequence of this fungus (Martínez et al. 2004). All these approaches to obtain mating type genes are very laborious and often also difficult.

To understand the evolution of mating type loci and the different breeding systems in the basidiomycetes, genes from more species need to be cloned and analyzed. In higher basidiomycetes, a highly conserved gene for a mitochondrial intermediate peptidase (*mip*) is found directly next (<1 kb) to the *A* mating type genes (James et al. 2004a). This gene has successfully been employed in positional cloning of *A* mating type genes from *Coprinopsis scobicola* (= *Coprinus bilanatus*; Kües et al. 2001), *Pleurotus djamor* (James et al. 2004b), and *Coprinellus disseminatus* (= *Coprinus disseminatus*; James 2003).

Fragments with *B* mating type gene sequences from *P. djamor* were initially identified through PCR using two pairs of degenerate primers, showing that such a PCR approach can be successful (James et al. 2004b). Another approach for isolating *B* mating type genes makes use of positional cloning. Previous studies indicated that in the ascomycete *Pneumocystis carini* (Smulian et al. 2001) and in the basidiomycetes *Cryptococcus neoformans* (Lengeler et al. 2002) and *P. chrysosporium* (James 2003) the p21-activated kinase gene *cla4*/*ste20* is closely linked to pheromone and pheromone receptor genes. The *cla4*/*ste20* gene was cloned from *P. djamor* and shown to reside at a distance of about 28 kb from to a pheromone gene and a pheromone receptor gene (James et al. 2004b). Close linkage between a *cla4*/*ste20* gene and the *a* mating type locus of *U. maydis* has now also been reported (Leveleki et al. 2004), and this syntemic arrangement is also conserved in *C. cinerea* as observed in the genome sequence (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/). In conclusion, positional cloning through *cla4*/*ste20* appears to be a most promising strategy to obtain *B* mating type genes from many species.

5.3. Materials and methods

C. cinerea strains were grown on YMG/T medium or minimal medium and transformations performed as described by Granado et al. (1997). Monokaryons 218 (*A3*, *B1*, *trp-1.1,1.6*, *bad*), LN118 (*A42*, *B42*, *ade-2*, *trp-1.1,1.6*) and FA2222 (*A5*, *B6*, *acu-1*, *trp1.1,1.6*) and NA2 (*ΔA*, *B6*, *ade-8*, *trp1.1-1.6*) were used as recipients in transformation (Pardo 1995; Kertesz-Chaloupková et al. 1998; Kües et al. 2002b). Monokaryon MK-45 (*A43*, *B1*, *ade-8*) was used in mating (Kües et al. 2001b). Plasmid pCc1001 with the *C. cinerea trp1* wild-type gene (Binninger et al. 1987) served in selection of transformants in co-transformation experiments. The cloned *A* mating type mutant fusion gene *a2-1/d1-1* from *C. cinerea* (Kües et al. 1994), the wild-type *A* mating type gene *a2-1* from *C. cinerea* (Kües et al. 1992), the *B42* mating type genes from *C. cinerea* cloned in cosmid cJH8 (Halsall et al. 2000) and the *A* mating type genes from *C. scobicola* cloned in cosmid 28D4 (Kües et al. 2001a) were analyzed by transformation. Positive transformants were identified by clamp cell production, clamp cell fusion and mating reaction, respectively.

5.4. Results and Discussion

5.4.1. Functional analysis of cloned wild-type A mating type genes

Transformation systems that allow functional analysis of cloned potential mating type genes exist for only very few basidiomycetes. In the higher basidiomycetes, functions of cloned *A* and *B* genes from *C. cinerea* and *S. commune* have been tested in strains of the same species (for details see the reviews cited above). The first heterologous expression of *C. cinerea A* mating type genes was achieved in the closely related heterothallic fungus C. *scobicola* (Challen et al. 1993). Subsequently, function of *A* mating type genes from *C. scobicola* in *C. cinerea* was also demonstrated. *C. scobicola A* mating type genes are active with resident genes from various *C. cinerea* monokaryons (Kües et al. 2001a; Fig. 3). Transformation experiments with heterologous genes from other species suggest that *C. cinerea* monokaryon 218 (*A3*, *B1*, *trp-1.1,1.6*) reacts best in tests with *A* mating type genes from foreign species (Srivilai, unpublished observation; James et al., in preparation). Different scenarios can be tested in strains that have endogenous mating type genes: the general function of an entire cloned *A* mating type locus within the heterologous species (Kües et al. 2001a; Fig. 3) or the behavior of individual *A* genes and whether they interact with foreign *A* genes resident in the new host (Challen et al. 1993).

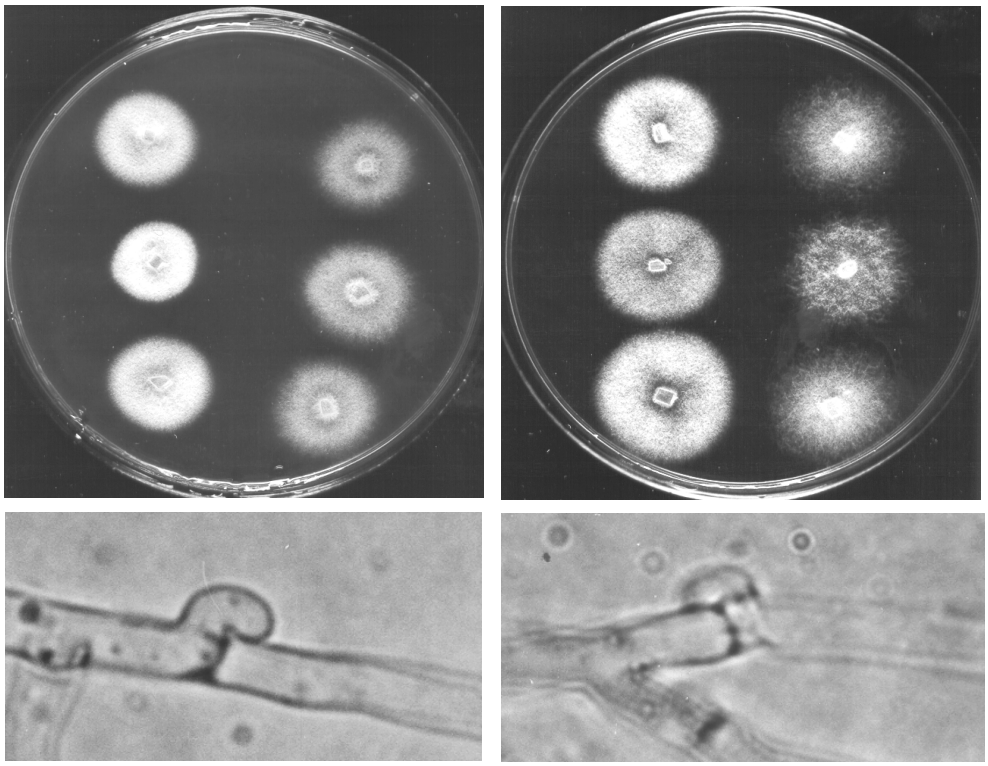


Figure 3. Expression of *C. scobicola A* mating type genes from cosmid 28D4 in *C. cinerea* monokaryon LN118 (*A42*, *B42*, *ade-2*, *trp-1.1, 1.6*) and FA2222 (*A5*, *B6*, *acu-1*, *trp1.1,1.6*). A and B show typical colony morphologies of densely grown monokaryotic clones (left) transformed with the *trp1*$^{+}$ plasmid pCc1001 (Binninger et al. 1987) and of less dense, more fluffy clones co-transformed with pCc1001 and cosmid 28D4 with *A* mating type genes from *C. scobicola* (right). C and D show unfused clamp cells found at hyphal septa in the *A*-activated transformants of LN118 and FA2222, respectively.

5.4.2. Functional analysis of cloned mutant *A* mating type genes

In *C. cinerea*, mutations in the *A* mating type locus are known that lead to self-compatibility. These mutations originate from large deletions of mating type DNA leading to in frame-fusions of an *HD2* and an *HD1* gene whose products normally do not interact. However, the product of the *HD2-HD1* fusion gene acts in the same manner as normal heterodimers of HD2 and HD1 proteins from different mating types. Activity but not self-compatibility of such fusion genes can be tested by clamp cell production in backgrounds of hosts with different mating type specificity. Self-compatibility can be shown by transformation into a wild-type self-background, i.e. by transformation into a monokaryon that carries the original unfused genes (Kües et al. 1994; Pardo et al. 1996). More elegant is the use of an *A*-null strain such as NA2 (Pardo 1995), where no resident *A* genes can interfere with action of the fusion gene (Fig. 4).

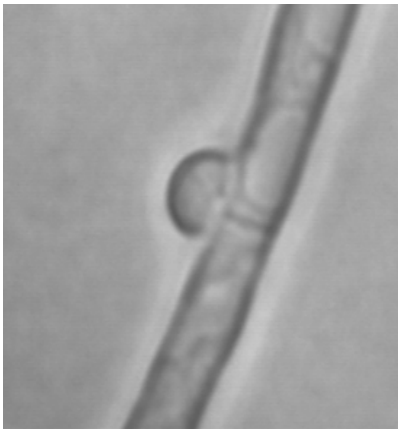


Figure 4. Clamp cell produced in *A* null mutant strain NA2 (*ΔA*, *B6*, *ade-8*, *trp1.1-1.6*) upon transformation with the self-compatible fusion gene *a2-1/d1-1* generated by deletion from an *A6* wildtype locus (Kües et al. 1994; photo by E. Polak)

The bipolar species *C. disseminatus* is found to have unlinked loci for homeodomain transcription factors and for pheromone genes and pheromone receptors, respectively, suggesting that one of these loci is either inactive or self-compatible (James et al., in preparation). Transformation into *C. cinerea* NA2 can help to clarify whether for example the locus for homeodomain transcription factors confers self-compatibility. Likewise, in strain NA2 one might test self-compatibility of genes for homeodomain transcription factors cloned from homothallic species. For example, self-compatibility of *A* mating type genes from the understood homothallic species *P. chrysosporium* (Martínez et al. 2004) might be tested in mutant NA2. Moreover, co-transformation with *A* genes from two different mating types are possible in this strain enabling analysis of the interaction of two distinct genes at a time (Polak 1999; Kües et al. 2001b).

5.4.3. Functional analysis of cloned *B* mating type genes

Functionality of *B* mating type genes can be tested in *C. cinerea* by transformation into a suitable monokaryon with different *B* mating type genes and subsequent mating of the transformants to another monokaryon carrying the same resident *B* mating type locus but a different *A* mating type locus (O´Shea et al. 1998). In this study for example, *B42* mating type transformants of the *A3*, *B1* monokaryon 218 were identified in crosses with *A43*, *B1* monokaryon MK-45. In monokaryon 218, it is also possible to recognize functional expression of compatible *B* mating type genes by a specific colony morphology on complete medium. Positive transformants show retarded colony growth, produce little aerial mycelium and have an irregular hyphal morphology, resembling the so-called “flat” phenotype of *Schizophyllum commune* strains with an activated *B* mating type pathway (Kües et al. 2002b; Fig. 5).

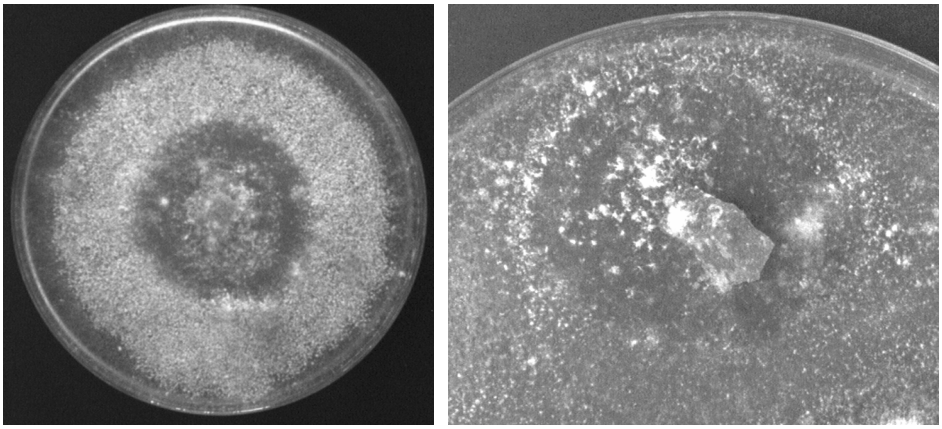


Figure 5. *B42* mating type transformants of monokaryon 218 produce no or only little aerial mycelium on YMG/T complete medium. Such transformants often need two to three weeks and more time of incubation at 37°C to completely overgrow a whole agar plate. In comparison, the untransformed monokaryon takes 7 days for full growth on a 9cm ∅ YMG/T plate.

When simultaneously transformed with an *A* mating type gene of a specificity compatible to that already present in the monokaryotic strain, clamp cell production apical to a septum is induced by action of the *A* mating type genes and *B*-induced clamp cell fusion to the subapical hyphal cell can be observed. Usually in the mycelium of such transformants, more often than formation of fused clamp cells, *B*-regulated formation of subapical pegs is detectable on septa with non-fused clamp cells (Kües et al. 2002b; Badalyan et al. 2004; Fig. 6).

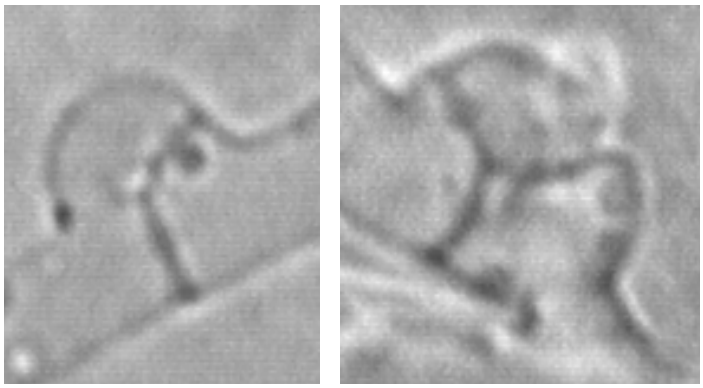


Figure 6. Fused clamp cell (left) and unfused clamp cell accompanied by a subapical peg (right) produced in monokaryon 218 upon co-transformation with compatible *A* and compatible *B*.

Similar observations in monokaryon 218 can be made when transforming heterologous *B* genes from other species into the strain (Srivilai, unpublished). A *B* null mutant from *C. cinerea* so far is unfortunately not available to test self-compatibility of cloned homologous and heterologous *B* mating type genes.

5.5. Conclusions

Mating type genes have now been cloned from several different basidiomycete species. Cloning of mating type loci from further species is now possible by positional cloning. The function of these mating type genes from other species can be addressed by transformation into *C. cinerea*. Our work showed that at least in some cases, foreign mating type genes are functionally expressed. Foreign gene products appear to interact with those from resident genes. Self-compatible *A* mating type genes and specific combinations of pairs of *A* mating type genes can be analyzed in an *A* null background of *C. cinerea*. Activation of the *B* mating type pathway through transformation can cause severe phenotypes. For the first time, foreign *B* mating type genes are also seen to function in *C. cinerea*.

5.6. Acknowledgements

PS and WC hold PhD scholarships of the Mahasarakham University (Thailand) and the University of Technology Lanna Phitsanulok Campus (Thailand), respectively. The Göttingen laboratory is supported by the Deutsche Bundesstiftung Umwelt (DBU).

5.7. References

Alic. M., C. Letzring, and M. H. Gold, 1987 Mating system and basidiospore formation in the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Appl. Environ. Microbiol. **53:** 1464-1469.

Badalyan, S. M., E. Polak, M. Aebi, and U. Kües, 2004 Role of peg formation in clamp cell fusion of homobasidiomycete fungi. J. Basic Microbiol. **44:** 167-177.

Binninger, D. M., C. Skrzynia, P. J. Pukkila, and L. A. Casselton, 1987 DNA-mediated transformation of the basidiomycete *Coprinus cinereus*. EMBO J. **6:** 835-840.

Casselton, L. A., and N. S. Olesnicky, 1998 Molecular genetics of mating recognition in basidiomycete fungi. Microbiol. Mol. Biol. Rev. **62:** 55-70.

Casselton, L. A., and M. P. Challen, 2005 The mating type genes of the basidiomycetes in *The Mycota*, Vol. I, Ed.2., edited by U. Kües and R. Fischer. Springer, Heidelberg, Germany, in press.

Challen, M. P., T. J. Eliott, U. Kües, and L. A. Casselton, 1993 Expression of *A* mating type genes of *Coprinus cinereus* in heterologous basidiomycete host. Mol. Gen. Genet. **241:** 474-478.

Giasson, L., C. A. Specht, C. Milgrim, C. P. Novotny, and R. C. Ullrich, 1989 Cloning and comparison of *Aα* mating type alleles of the basidiomycete *Schizophyllum commune*. Mol. Gen. Genet. **218:** 72-77.

Granado, J. D., K. Kertesz-Chaloupková, M. Aebi, and U., Kües, 1997 Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. Mol. Gen. Genet. **256:** 28-36.

Halsall, J. R., M. J. Milner, and L. A. Casselton, 2000 Three subfamilies of pheromone and receptor genes generate multiple *B* mating specificities in the mushroom *Coprinus cinereus*. Genetics **154:** 1115-1123.

Hiscock, S. J., and U. Kües, 1999 Cellular and molecular mechanisms of sexual incompatibility in plants and fungi. Int. Rev. Cytol. **193:** 165-295.

James, T. Y., 2003 *The Evolution of Mating-type Genes in the Mushroom Fungi (Homobasidiomycetes)*. Ph.D. thesis, Duke University, Durham, North Carolina.

James, T. Y., U. Kües, S. A. Rehner, and R. Vilgalys, 2004 Evolution of the gene encoding mitochondrial intermediate peptidase and its cosegregation with the *A* mating-type locus of mushroom fungi. Fungal Genet. Biol. **41:** 381-390.

James T. Y., U. Kües, S. A. Rehner, R. Vilgalys, 2004b The genetic structure and diversity of the *A* and *B* mating-type genes from the tropical oyster mushroom, *Pleurotus djamor*. Fungal Genet. Biol. **41:** 813-825.

Kertesz-Chaloupková, K., P. J. Walser, J. D. Granado, M. Aebi, and U. Kües, 1998 Blue light overrides repression of asexual sporulation by mating type genes in the basidiomycete *Coprinus cinereus*. Fungal Genet. Biol. **23:** 95-109.

Kothe, E., 2001 Mating-type genes for basidiomycete strain improvement in mushroom farming. Appl. Microbiol. Biotechnol. **56:** 602-612.

Kües, U., 2000. Life history and developmental processes in the basidiomycete *Coprinus cinereus*. Microbiol. Mol. Biol. Rev. **64:** 316-353.

Kües, U., W. V. J. Richardson, A. M. Tymon, B. Göttgens *et al.*, 1992 The combination of dissimilar alleles of the $A\alpha$ and $A\beta$ gene complexes, whose proteins contain homeodomain motifs, determines sexual development in the mushroom *Coprinus cinereus*. Genes Dev. **6:** 568-577.

Kües, U., B. Göttgens, R. Stratmann, W. V. J. Richardson, S. F. O Shea *et al.*, 1994 A chimeric homeodomain protein causes self compatibility in the mushroom *Coprinus cinereus*. EMBO J. **13:** 4054-4059.

Kües, U., T. Y. James, R. Vilgalys, M. P. Challen, 2001 The chromosomal region containing *pab-1*, *mip*, and the *A* mating type locus of the secondary homothallic homobasidiomycete *Coprinus bilanatus*. Curr. Genet. **39:** 16-24.

Kües, U., M. J. Klaus, E. Polak, M. Aebi, 2001 Multiple cotransformations in *Coprinus cinereus*. Fungal Genetics Newsl. **48:** 32-34.

Kües, U., E. Polak, A. P. F. Bottoli, M. Hollenstein, P. J. Walser *et al.*, 2002 Vegetative development in *Coprinus cinereus*, pp. 133-164 in *Molecular Biology of Fungal Development*, edited by H. D. Osiewacz, Marcel Dekker, New York.

Kües, U., P. J. Walser, M. J. Klaus, and M. Aebi, 2002 Influence of activated *A* and *B* mating type pathways on developmental processes in the basidiomycete *Coprinus cinereus*. Mol. Gen. Genom. **268:** 262-271.

Kües, U., M. Künzler, A. P. F. Bottoli, P. J. Walser, J. D. Granado *et al.*, 2004 Mushroom development in higher basidiomycetes; implications for human and animal health, pp. 431-470 in *Fungi in Human and Animal Health*, edited by R. K. S. Kushwaha. Scientific Publishers, Jodhpur, India.

Lengeler, K., B. D. S., Fox, J. A. Fraser, A. Allen, K, Forrester *et al.*, 2002 The mating type locus of *Cryptococcus neoformans*: a step in the evolution of sex chromosome. Eukaryot. Cell **1:** 704-718.

Leveleki L., M. Mahlert, B. Sandrock, and M. Bölker, 2004 The PAK family kinase Cla4 is required for budding and morphogenesis in *Ustilago maydis*. Mol. Microbiol. **54:** 396-406.

Martinez, D, L. F. Larrondo, N. Putnam, M. D. Gelpke, K. Huang *et al.*, 2004. Genome sequence of the lignocellulose degrading fungus *Phanerochaete chrysosporium* strain RP78. Nat. Biotechnol. **22:** 695-700.

Mutasa, E. S., A. M. Tymon, B. Göttgens, F. M. Mellon, P. F. R. Little *et al.*, 1990 Molecular organisation of an *A* mating type factor of the basidiomycete fungus *Coprinus cinereus*. Curr. Genet. **18:** 223-229.

O Shea, S. F., P. T. Chaure, J. R. Halsall, N. S. Olesnicky, A. Leibbrandt *et al.*, 1998 A large pheromone and receptor gene complex determines multiple *B* mating type specificities in *Coprinus cinereus*. Genetics **148:** 1081-1090.

Pardo, E. H., 1995 *Organization of the A Mating Type Locus of Coprinus cinereus*. Ph.D. thesis, University of Oxford, Oxford, UK.

Pardo, E. H., S. F. O'Shea, and L.A. Casselton, 1996 Multiple versions of the *A* mating type locus of *Coprinus cinereus* are generated by three paralogous pairs of multiallelic homeobox genes. Genetics **144:** 87-94.

Polak, E., 1999 *Asexual Sporulation in the Basidiomycete Coprinus cinereus*. Ph.D. thesis, ETH Zurich, Zurich, Switzerland.

Quintanilha and Pinot-Lopes. 1950 Aperçu sur l'état actuel de nos connaissances concernant la 'conduite sexuelle' des espèces d'Hymenomycetes. Bol. Soc. Broteriana. **24:** 115-290.

Riquelme, M., M. P. Challen, L. A. Casselton, and A. J. Brown, 2005 The origin of multiple *B* mating specificities in *Coprinus cinereus*. Genetics **170:** 1105-1119.

Smulian, A. G., T. Sesterhenn, R. Tanaka, and M. T. Cushion, 2001 The *ste3* pheromone receptor gene of *Pneumocysti carini* is surrounded by a cluster of signal transduction genes. Genetics **157:** 991-1002.

Specht, C. A., 1995 Isolation of the *Bα* and *Bβ* mating-type loci of *Schizophyllum commune*. Curr. Genet. **28:** 374-379.

Whitehouse, H. L. K., 1949 Multiple allelomorph heterothallism in the fungi. New Phytol. **48:** 212-244.

CHAPTER 6

A mating type genes repress oidia production of *Coprinopsis cinerea*

6.1. Abstract

The *A* mating type locus of *Coprinopsis cinerea* consists of two *A* mating type subloci i.e., the $A\beta$ and $A\alpha$ subloci. Each sublocus contains genes with multiple alleles that encode two types of homeodomain transcription factors, namely *HD1* and *HD2*. In sexual reproduction of *C. cinerea*, HD1 and HD2 proteins from one mating type have to interact with HD2 and HD1 proteins of another mating type in order to form heterodimeric protein complexes, which act in induction of clamp cell formation and initiation of fruiting body development and as repressors of asexual sporulation (odiation). This study focused on the functions of single and of compatible and incompatible *A* mating type genes in the monokaryotic *A*-null strain NA2 (*Anull B6*). Single, and pairs of compatible, and incompatible *A* mating type genes were transformed into the *A*-null strain. Compatible *A* mating type genes displayed a crucial role to negatively regulate oidia production under dark growth conditions. An incompatible combination of an *HD1* and an *HD2* gene was not effective. Of singly transformed genes, transformants of the *HD1* gene *a1-3* all had a strongly reduced spore number per plate in the dark.

6.2. Introduction

Coprinopsis cinerea is a basidiomycete that consists of two types of mycelia, namely the monokaryons, and the dikaryons. The monokaryon has simple septa and forms constitutively within the vegetative mycelium asexual spores (oidia) on specialized aerial hyphae (oidiophores). The dikaryon is generated by mating of two compatible monokaryons. The dikaryon has clamp cells at each septum and develops fruiting bodies under appropriate environmental conditions (low light intensity in a day/night rhythm, 80-90% humidity, temperatures between 25-28 °C and depletion of nutrients). The development of the dikaryon is controlled by the *A* and *B* mating type genes. By mating of two compatible monokaryons, the dikaryon derives two distinct haploid nuclei containing the genes of different *A* and *B* mating type specificities from the parental monokaryons. In the maintenance of the dikaryon, the *A* mating type genes control the nuclear pairing, initiation of clamp cell formation, synchronization of nuclear division and septation (Kües et al. 1998; Kües 2000). As a result of these processes, one of the daughter nuclei from the nuclear divisions is entrapped in the clamp cell until the clamp cell fuses with the sub-apical cell, thereby forming a clamp connection. The clamp cell does not fuse directly to the sub-apical cell, but to a peg formed at the sub-apical cell. Upon clamp cell – peg fusion, the entrapped nucleus migrates from the clamp cell into the sub-apical cell where it pairs with the haploid nucleus of the other mating type (Casselton and Kües 1994; Casselton and Olesnicky 1998; Badalyan et al. 2004). The *B* mating type genes regulate the sub-apical peg formation and clamp cell fusion (Kües 2000; Badalyan et al. 2004; Riquelme et al. 2005). Both the *A* and the *B* locus contain multiple allelic genes. It is estimated that there are about 160 different *A* mating type specificities, and 80 different *B* mating type specificities in nature (Raper 1966).

The *A* mating type locus encodes two distinct types of homeodomain transcription factors, namely HD1 and HD2. For regulation of dikaryon development, HD1 and HD2 proteins from one nucleus must interact with HD2 and HD1 proteins of another compatible nucleus, respectively i.e., HD1-1 X HD2-2 or HD2-1 X HD1-2 (-1 and -2 references to different allelic specificities). Furthermore, the results from gene transformation experiments with cloned *A* mating type genes in compatible monokaryons indicated that the *A* mating type genes repress oidiation in the dikaryon (Tymon et al. 1992; Kües et al. 1994, 2002; Kertesz-Chaloupková et al. 1998). Furthermore, *A*on transformants of the monokaryon 218 forms enlarged numbers of chlamydospores, hyphal knots and sclerotia. When a light signal is given, fruiting body primordia develop but they arrest in development at the stage of karyogamy (Kües et al. 2002). Whilst transformation work repeatedly showed that reactions between compatible HD1 and HD2 proteins regulate various steps in development, not much is known about whether there is a function of the proteins before mating.

Pardo (1995) produced an *A*-null strain of *C. cinerea* by knocking out the complete *A* locus and showed in this way, that the *A* mating type genes are not essential. Polak (1999) transformed a single *HD1* and a single *HD2* gene into the strain and observed a light – controlled reaction in oidiation in one transformant of an *HD1* gene. To follow up this further, in this work, I transformed single *HD1* and single *HD2* genes, incompatible pairs of an *HD1* and *HD2* genes, and compatible pairs of *HD1* and *HD2* genes into the *A*-null strain made by Pardo (1995) and followed up the behaviour of transformants in asexual spore formation.

6.3. Materials and methods

6.3.1. *C. cinerea* strains, plasmids and transformation

C. cinerea strains used in this study were the *A*-null strain (*Anull B6 trp1.1,1.6*), constructed by Pardo (1995), and monokaryons JV6 (*A42 B42*), FA2222 (*A5 B6, acu-1, trp1.1,1.6*), PS001-1 (*A42 B42*), PS002-1 (*A3 B1*) and Okayama 7 (*A43 B43*) (chapter 3 of this thesis; Kertesz-Chaloupková et al. 1998; May et al. 1991). Strain NA2 was transformed following the protocol of Granado et al. (1997). 1 μg DNA of a plasmid carrying an *A* mating type genes were co-transformed with 1 μg of pCc1001 carrying the *trp-1* wild type gene (Binninger et al. 1987). A control transformation was preformed with pCc1001 alone.

Plasmids with *A* mating type genes were as follows: pUK2 contains a 2.1 kb S*al*I fragment with the *a2-1* gene isolated from the *A42* mating type locus (Kües et al. 1992), pE12P5-3 carries gene *a1-2* from the *A43* mating type locus (May et al. 1991). pCR8 carries a 4.3 kb *Sal*I-*Pst*I fragment with the *a1-3* gene of the *A5* mating type locus and pCR7 carries a 4.3 kb *Bam*HI- *Pst*I fragment with the *a2-3* gene of the *A5* mating type locus (Pardo et al. 1996).

To identify the presence of *A* mating type gene, single gene transformants and of transformants with the set of incompatible genes (i.e. *a1-3* and *a2-3*) were crossed with suitable monokaryotic tester strains listed above (Walser et al. 2001). Clamp cell formation upon dikaryon formation was investigated under the light microscope. Transformant of sets of compatible *HD1* and *HD2* genes (*a1-2+a2-1, a1-2+a2-3* and *a1-3+a2-1*) were recognized by formation of unfused clamp cells directly on the transformants.

6.3.2. Oidiation test

The standard oidiation test was conducted following Kertesz-Chaloupková et al. (1998). The transformants were grown on YMG/T medium (Granado et al. 1997) in light-proofed ventilated box for 4 days (three inoculums per plate), fully-grown cultures were then transferred to light (light spectrum of 295-780 nm) for 5 days whilst another subset was further kept in the dark. Oidia were

harvested in 10 ml water by scraping the agar surface and mycelia and oidia separated by filtration with a glass funnel containing glass wool. The number of oidia was calculated from counting spores with a Thoma chamber (Kertesz-Chaloupková et al. 1998).

6.4. Results

Various plasmids with *A* mating type genes were transformed either singly or in compatible or incompatible pairs into protoplasts of the *C. cinerea* strain *A*-null strain NA2 (*Anull B6, trp1.1, 1.6*) which was constructed by Pardo (1995). Transformants obtained on regeneration medium were then transferred onto minimal medium (MM) (Granado et al. 1997) as stock cultures and incubated at 37 °C for 36 hours. Transformants which were obtained from the transformation of a mixture of compatible *A* mating type genes (*a1-2* and *a2-1, a1-2* and *a2-3* and *a1-3* and *a2-1*) were then investigated for the clamp cell formation directly under the light microscope. In contrast, the transformants which were obtained by transformation of only a single and or of two incompatible *A* mating type genes were checked for promoting clamp cell formation in dikaryons by crossing them with the suitable tester strains (see the list in Material and Method; Fig. 1). The frequencies of positive transformants of *A* mating type genes amongst the complete set of *trp-1*$^+$ transformants obtained are shown in Table 1. In case of the transformation with the single *A* mating type genes, the frequencies of co-transformation of *A* mating type genes together with *trp-1*$^+$ was from 19% to 43 % (Table 1). The frequencies of transfer of both mating type genes in the co-transformation of a combination of an *HD1* and an *HD2* gene together with the *trp1+* was always lower, between 7 % and 16 % (Table 1). These values are within the rage of co-transformation of single mating type genes and combination of mating type gene as described before (Kües et al. 2001). In co-transformation experiments with pairs of *A* mating type genes and the *trp1+* gene, transfer rates of the individual genes were found to be in the range of 60-70 % of all *trp1*$^+$ transformants and the co-transformation frequencies of the *trp+* gene and two different *A* mating type genes were in the range of 5-35 % (Kües et al. 2001). Thus, total transformation rates of each one of the two mating type genes in the co-transformation experiments done with three plasmids in this work were possibly much better than the transfer rates of both mating type genes given in Table 1. However, in this work only in one case (co-transformation experiment with the incompatible *HD1* and *HD2* gene pair *a1-3* and *a2-3*) also the transfer rates of the individual mating type genes were determined. In total, the individual co-transformation frequency of the *HD1* gene *a1-3* was 8% and the individual co-transformation frequency of the *HD2* gene *a2-3* was 13% (see footnote to Table 1). Thus, in this case, individual transfer rates were only slightly higher than the transfer rates of all plasmids.

Table 1 Co-transformation of the *trp1+* containing plasmid pCc1001 with single *A* mating type genes and combination of *A* mating type genes into the *C. cinerea A*-null knockout strain NA2

***A* mating type genes**	**Total of *trp-1+* transformants tested**	***A* mating type gene (s) present in the positive *trp+* transformants**	**Frequency of co-transformation**
- (**)	30	-	-
a1-2	33	7	21%
a2-1	40	17	43%
a1-3	32	6	19%
a2-3	30	7	23%
a1-2+a2-1	50	8	16%
a1-2+a2-3	40	5	13%
a1-3+a2-1	30	4	13%
a1-3+a2-3	101(*)	7	7%

* Of the 101 transformants tested in total, 8 clones had the *a1-3* gene (8%) and 13 clones had the *a2-3* gene (13%); compare Fig. 1.
** pCc1001 control

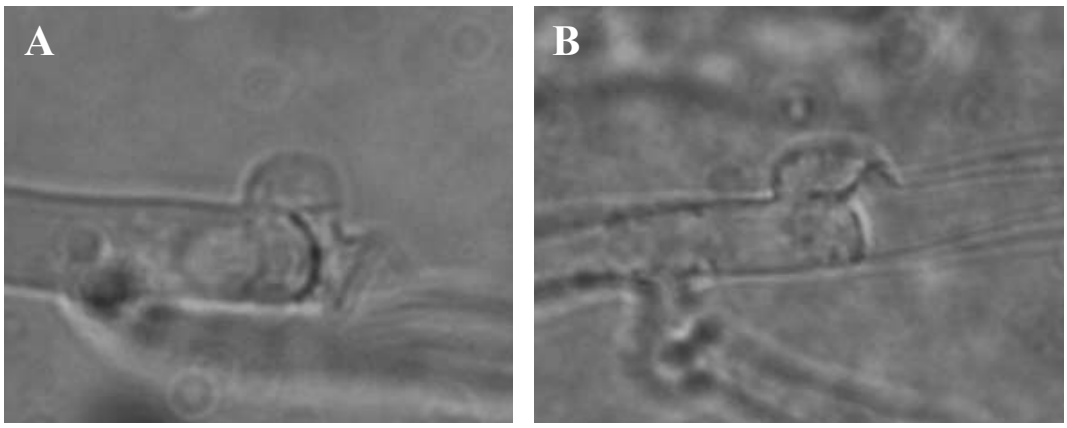


Figure 1. Representative microscopic photographs of clamp cell formation investigated by crossing a *trp+* clone from the transformation with the combination of *A* mating type genes *a1-3* and *a2-3* to the *A42* monokaryon JV6 (**A**), and to the *A43* monokaryon Okayama 7 (**B**). In the *A42* locus with gene *a2-1*, there is only a compatible partner to gene *a1-3* and in the *A43* locus with gene *a1-2*, there is only a compatible partner to *a2-3* (Pardo et al. 1996). Clamp cell formation, upon both crosses therefore proofs the successful transfer of both *A* mating type genes.

6.4.1. Growth of mycelium of transformants

The growth of the mycelium of transformants containing single *A* mating type genes showed neglectable differences in morphology from each to other and from pCc1001 transformants. Similarly, the morphology and the hyphal widths of the transformants containing the combination of incompatible *A* mating type genes were not different from transformants containing a single *A* mating type gene (Fig. 2 and Fig. 3). In contrast, the transformants obtained from the transformation with pairs of compatible *A* mating type genes had a slightly different morphology when compared with those of another transformants due to formation of clamp cells (Fig. 2 and Fig. 3).

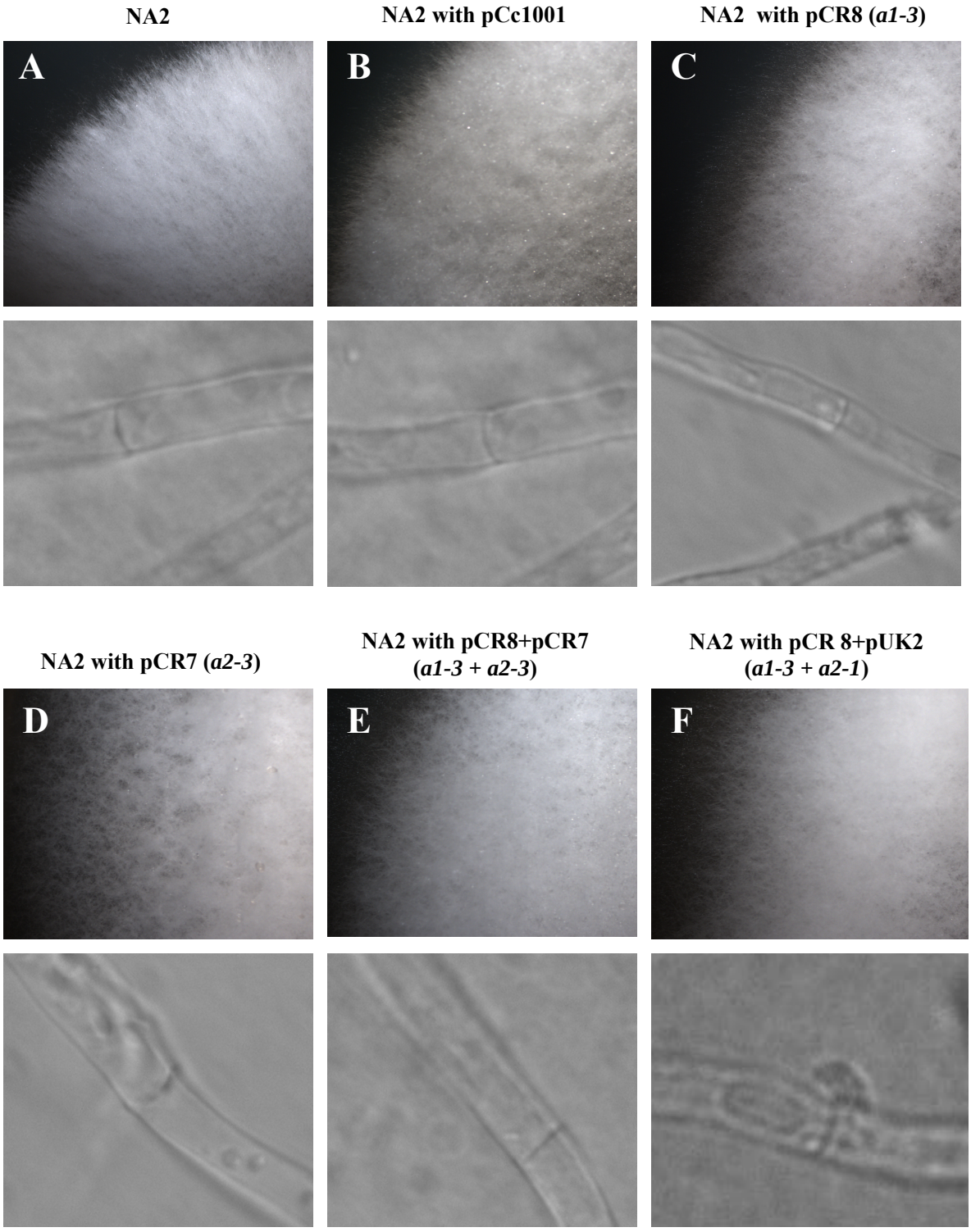


Figure 2. Mycelial phenotype (top) and the hyphal morphology (bottom) of the untransformed strain NA2 (**A**), and of the NA2 strain containing plasmid pCc10001 carrying the tryptophan gene *trp1+* (**B**), of strain NA2 containing in addition to pCc1001 plasmid pCR8 carrying the single *A* mating type *a1-3* gene (**C**), of strain NA2 containing in addition to pCc1001 plasmid pCR7 carrying the single *A* mating type *a2-3* gene (**D**), of strain NA2 containing in addition to pCc1001 the co-transformed plasmids pCR8 and pCR7 carrying the two *A* mating type genes *a1-3* and *a2-3*, respectively (**E**), and of strain NA2 containing in addition to pCc1001 the co-transformed plasmids pCR8 and pUK2 carrying the two *A* mating type genes *a1-3* and *a2-1*, respectively (**F**).

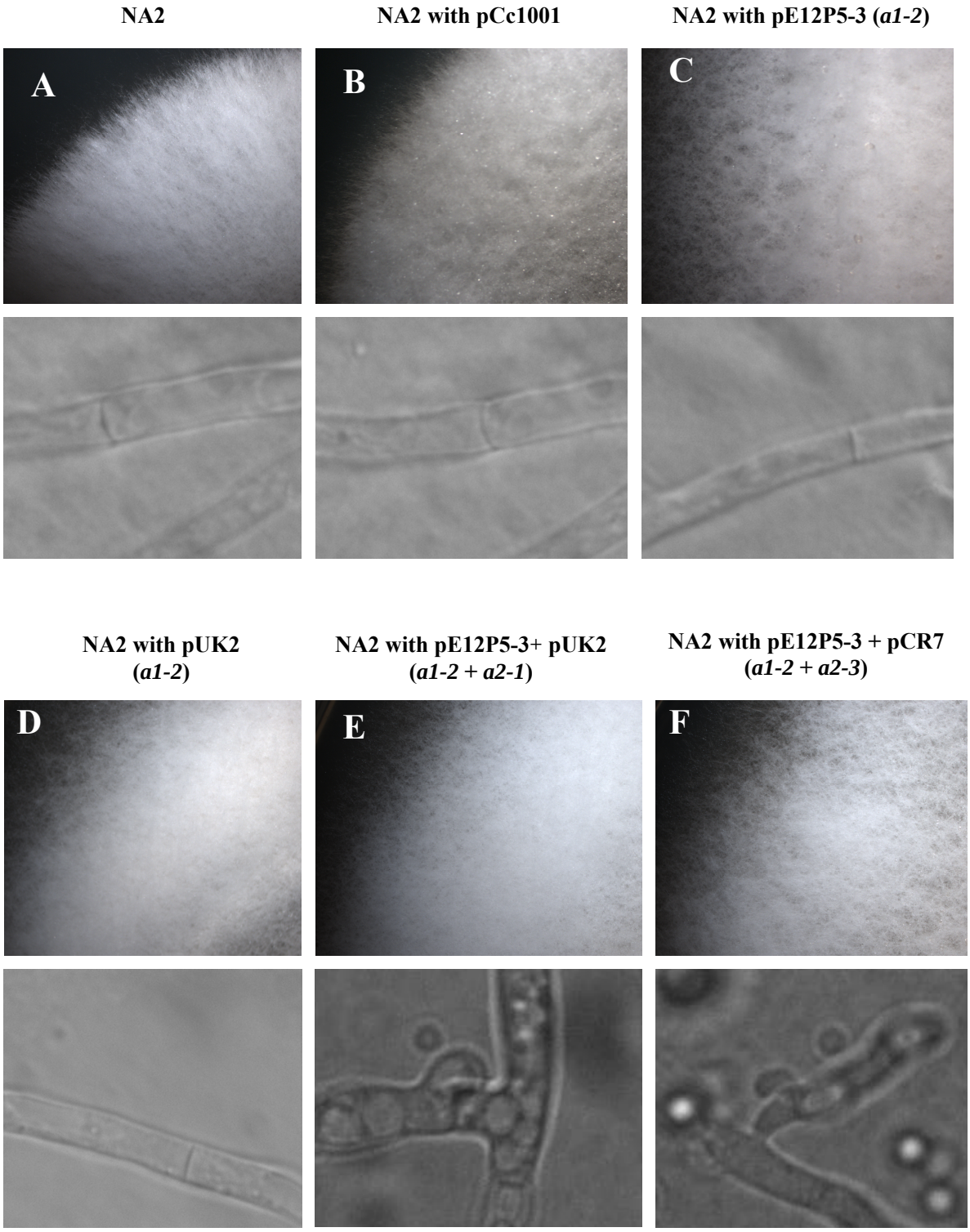


Figure 3. Photographs showing the mycelial phenotype (top) and the hyphal morphology (bottom) of the untransformed stain NA2 (**A**), and of the strain transformant only with plasmid pCc10001 carrying the *trp1+* gene (**B**), of strain NA2 containing in addition to pCc1001 plasmid pE12P5-3 carrying the single *A* mating type gene *a1-2* (**C**), of strain NA2 containing in addition to pCc1001 plasmid pUK2 carrying the single *A* mating type gene *a2-1* (**D**), of strain NA2 containing in addition to pCc1001 the co-transformed plasmids pE12P5-3 and pUK2 carrying the *A* mating type genes *a1-2* and *a2-1*, respectively (**E**), and of strain NA2 containing in addition to pCc1001 the co-transformed plasmids pE12P5-3 and pCR7 carrying the *A* mating type genes *a1-2* and *a2-3*, respectively (**F**).

6.4.2. *A* mating type genes affect asexual spore (oidia) production

The effects of *A* mating type genes on asexual spore (oidia) formation was characterized by using the *A*-null strain NA2 transformed either with a single, or a pair of compatible or a pair of incompatible *A* mating type genes. Sets of fully grown cultures were kept for 5 days either in constant dark or in constant light at 37°C. At day 5, the oidia were harvested from each culture. The numbers of oidia were then counted by using the method described by Kertesz-Chaloupková et al. (1998). The results show that under light, all transformants had more spores than the parallel cultures kept in the dark (Fig. 4).

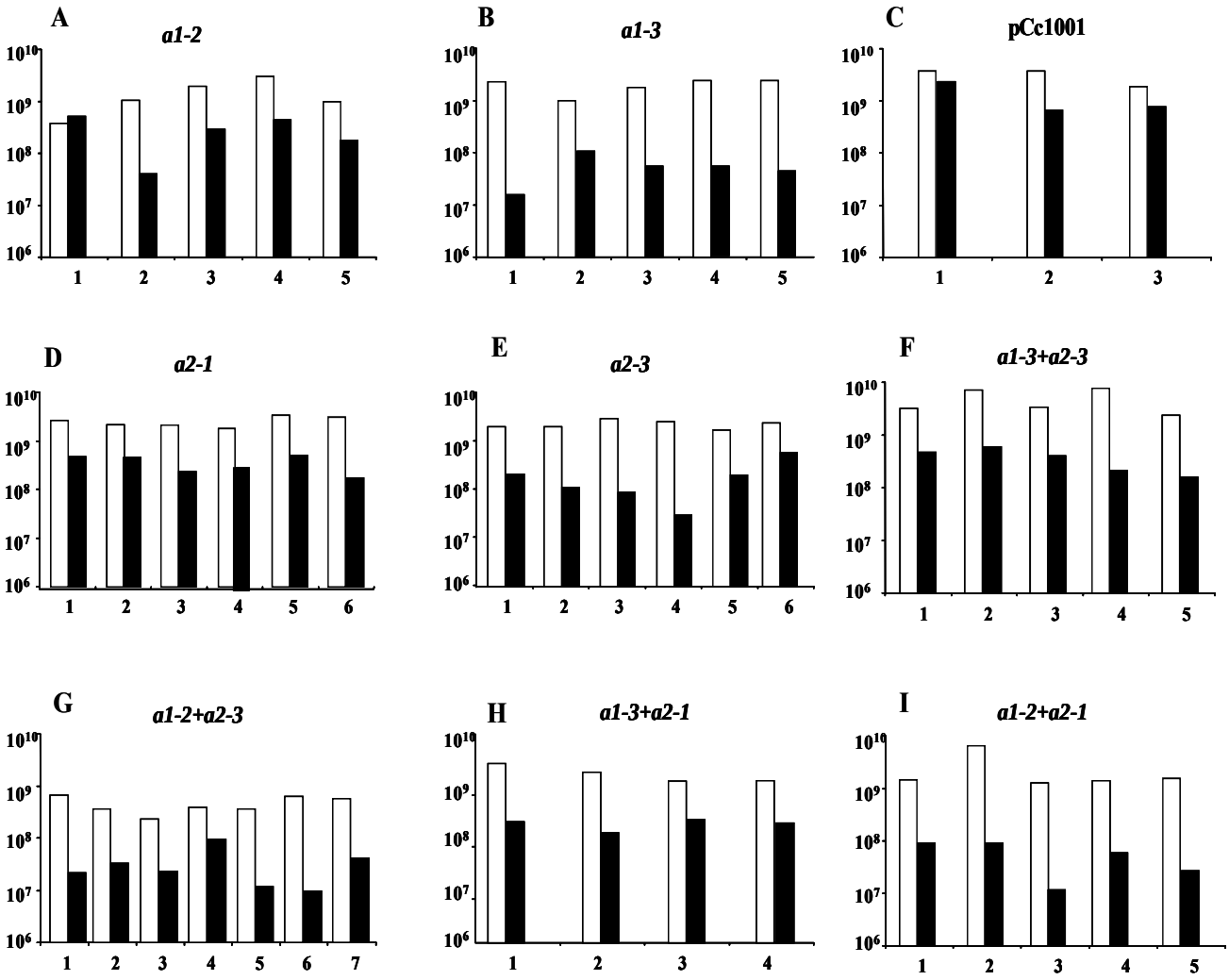


Figure 4. Comparison of the number of oidia produced per plate by individual transformants at 37 °C during growth for five days in constant dark and in constant light. Sporulation by transformants of a single *HD1* are shown in **A** and **B**, transformants of a single *HD2* gene in **D** and **E**, transformants of pairs of compatible *HD1* and *HD2* genes in **G**, **H** and **I**, and of transformants of an incompatible of of *HD1* and *HD2* genes in **F**. For control, pCc1001 transformants were used (**C**). (□) represents the number of oidia in light and (■) represents the number of oidia in dark.

Transformants of the compatible combinations *a1-2* and *a2-3*, and *a1-2* and *a2-1* had a lower spore production in the dark and a not full release of repression of oidiation in the light, as to be expected from strong transformants of every combination of compatible *HD1* and *HD2* genes (Fig. 4, G and I), (Kertesz-Chaloupková et al. 1998; Kües et al. 2002). The oidiation phenotype of the third combination of a compatible *HD1* and *HD2* gene pair (*a1-3* and *a2-1*) was not as pronounced (Fig. 4 H), suggesting a possible weak interaction between the two gene products. From the single genes, most interesting was the *HD1* gene *a1-3* (Fig.4B). All five tested transformants had only low levels of spores in the dark (spore numbers of $1x10^7$ to $6x10^7$) and a high increase in sporulation in light to normal monokaryon numbers (>109 spores per plate; compare Kertesz-Chaloupková et al. 1998). Amongst the *a1-2* transformants and the *a2-3* transformants, there were also each time one transformant with such a low number of spores in the dark and such a high increase in spore numbers in the light (Fig. 4A and Fig. 4E).

6.5. Discussion

The *C. cinerea* (HD1 and HD2) homeodomain transcription factors are encoded by the *A* mating type genes, and these transcription factors regulate the sexual development on the dikaryon (Casselton and Olesnicky 1998; Hiscock and Kües 1999; Kües et al. 2002; Kües 2000). The presence of compatible *A* mating type genes induced formation of clamp cells in the A*null* strain NA2, as to be expected from the results of previous transformation work on monokaryons FA2222 and 218 (Kües et al. 1998). The oidia formation in transformed *A*on/FA2222 and *A*on*/218* monokaryons was repressed under the dark condition. Most of the *A*on transformants produced oidia in numbers ranging only from 10^5 to 10^7 spores per YMG/T plate under the dark condition whilst simple *trp1*$^+$ transformants reach Numbers between 10^8 to 10^9 spores per YMG/T plate.

In this study, we found a somewhat more irregular behaviour of transformants of single and incompatible *A* mating type genes in oidia production in dark and light conditions. All transformants of the *a1-3* gene, one transformant of the *a1-2* gene and one transformant of the *a2-3* gene showed a strongly reduced oidia number in the dark compared to simple pCc1000 transformants and to most transformants of *HD1* gene *a1-2* and most transformants of the two *HD2* genes tested in this work (Fig. 4A and E). Polak et al. (1999) had reported before that a NA2 transformant of the *HD1* gene *a1-2* in her hands showed a 10 fold reduced oidia production in dark compared to simple pCc1001 and to *a2-1* transformants of strain NA2. Interestingly, in the work presented here, the HD1 protein which is encoded by gene *a1-3* appeared to give less oidia production in dark. This suggested that the HD1 protein repressed oidiation. The *C. cinerea* HD1 proteins contain a nuclear localization signal (NLS) required for the transfer of HD1-HD2 protein complexes into the nucleus (Spit et al. 1998). Although for clamp cell production DNA-binding of

HD1 proteins in not required (Kües et al. 1994; Asante-Owusu et al. 1995; Pardo et al. 1995), it is possible that the HD1 protein might be transferred on its own into the nucleus and bind at the DNA in absence of a compatible HD2 protein.

In any case, the majority of *HD1* gene transformants in this study appeared to effect oidia production in the dark, in contrast to the majority of transformant of *HD2* genes and one is tempered to believe that there is an effect on sporulation induced by these genes, as also suggested by the earlier study of Polak (1999). However, of each type of co-transformation more transformants are needed to be analyzed, and also in parallel cultures of the untransformed NA2 in order to exclude the possibility that reduction of oidia production in the dark is not just an intrinsic behaviour of the strain that appeared more or less in individual cultures.

6.6. References

Asante-Owusu, R. N., A. H. Banham, H. U. Böhnert, E. J. C., Mellor, and L. A. Casselton, 1996 Heterodimerization between two classes of homeodomain proteins in the mushroom *Coprinus cinereus* brings together potential DNA-binding and activation domains. Gene **172:** 25-31.

Binninger, D. M., C. Skrzynia, P. J. Pukkila, and L. A. Casselton, 1987 DNA-mediated transformation of the basidiomycete *Coprinus cinereus*. EMBO J. **6:** 835-840.

Casselton, L. A., and N. S. Olesnicky, 1998 Molecular genetics of mating recognition in basidiomycete fungi. Microbiol. Mol. Biol. Rev. **62:** 55-70.

Casselton, L. A., and U. Kües, 1994 Mating-type genes in homobasidiomycetes, pp. 307-322 in *The Mycota, Vol. I., Growth, Differentiation and Sexuality*, edited by J. G. H. Wessels and F. Meinhardt. Springer-Verlag KG, Berlin, Germany.

Hiscock, S. J., and U. Kües, 1999 Cellular and molecular mechanisms of sexual incompatibility in plants and fungi. Int. Rev. Cytol. **193:** 165-295.

Halsall, J. R., M. J. Milner, and L. A. Casselton, 2000 Three subfamilies of pheromone and receptor genes generate multiple *B* mating specificities in the mushroom *Coprinus cinereus*. Genetics, **154:** 1115-1123.

James, T. Y., P. Srivilai, U. Kües, and R. Vilgalys, 2006 Evolution of the bipolar mating system of the mushroom *Coprinellus disseminatus* from its tetrapolar ancestors involves loss of mating-type-specific pheromone receptor function. Genetics **172:** 1877-1891.

Kertesz-Chaloupková, K., P. J. Walser, J. D. Granado, M. Aebi, and U. Kües, 1998 Blue light overrides repression of asexual sporulation by mating type genes in the basidiomycete *Coprinus cinereus*. Fungal Genet. Biol. **23:** 95-109.

Kües, U., and L. A. Casselton, 1992 Homeodomains and regulation of sexual development in basidiomycetes. Trends Genet. **8:** 154-155.

Kües, U., B. Göttgens, R. Stratmann, W. V. J. Richardson, S. F. O'Shea *et al.*, 1994 A chimeric homeodomain protein causes self-compatibility and constitutive sexual development in the mushroom *Coprinus cinereus*. EMBO J. **13:** 4054-4059.

Kües, U., J. D. Granado, R. Hermann, R. P. Boulianne, K. Kertesz-Chaloupková *et al.*, 1998 The *A* mating type and blue light regulate all known differentiation processes in the basidiomycete *Coprinus cinereus*. Mol. Gen. Genet. **260:** 81-91.

Kües, U., M. J. Klaus, E. Polak, and M. Aebi, 2001 Multiple cotransformations in *Coprinus cinereus*. Fungal Genetics Newsl. **48:** 32-34.

Kües, U., 2000 Life History and Developmental Processes in the Basidiomycete *Coprinus cinereus*. Microbiol. Mol. Biol. Rev. **64:** 316-353.

Muraguchi, H., Y. Ito, T. Kamada, and S. A. Yanagi, 2003 Linkage map of the basidiomycete *Coprinus cinereus* based on random amplified polymorphic DNA and restriction fragment length polymorphisms. Fungal Genet. Biol. **40:** 93-102.

North, J., 1990 Linkage map of *Coprinus cinereus* Schaeff. Ex Fr. S.F.Gray, Ink cap. N=12, pp. 3.70-3.81 in *Genetic Maps: Locus Maps of Complex Genomes*, Ed.5., edited by S. J. O'Brien. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

O'Shea, S. F., P. T. Chaure, J. R. Halsall, N. S. Olesnicky, A. Leibbrandt *et al.*, 1998 A large pheromone and receptor gene complex determines multiple *B* mating type specificities in *Coprinus cinereus*. Genetics **148:** 1081-1090.

Pardo, E. H., 1995 *Organization of the A Mating Type Locus of Coprinus cinereus*. Ph.D. thesis, University of Oxford, Oxford, UK.

Pardo, E. H., S. F. O'Shea, and L. A. Casselton, 1996 Multiple versions of the *A* mating type locus of *Coprinus cinereus* are generated by three paralogous pairs of multiallelic homeobox genes. Genetics **144:** 87-94.

Polak, E., 1999 *Asexual Sporulation in the Basidiomycete Coprinus cinereus*. Ph.D. thesis, ETH Zurich, Zurich, Switzerland.

Raper, J. R., 1966 *Genetics of Sexuality in Higher Fungi*. Ronald Press, New York.

Srivilai, P., H. Muraguchi, and U. Kües, 2006 Construction of co-isogenic strains of *Coprinopsis cinerea* homokaryon *AmutBmut* with different wildtype mating type specificities. (In preparation).

Spit, A., R. H. Hyland, E. J. C. Mellor, and L.A. Casselton, 1992 A role for heterodimerization in nuclear localization of a homeodomain protein. Proc. Natl. Acad. Sci. USA **95:** 6228-6233.

Swiezynski, K. M., and P. R. Day, 1960 Heterokaryon formation in *Coprinus lagopus*. Genet. Res. **1:** 114-128.

Swiezynski, K. M., and P. R. Day, 1960 Migration of nuclei in *Coprinus lagopus*. Genet. Res. **1:** 129-139.

Tymon, A. M., U. Kües, W. V. J. Richardson, and L. A. Casselton, 1992 A fungal mating type protein that regulates sexual and asexual development contains a POU-related domain. EMBO J. **11:** 1805-1813.

CHAPTER 7

Evolution of the bipolar mating system of the mushroom *Coprinellus disseminatus* from its tetrapolar ancestors involves loss of mating-type-specific pheromone receptor function

Timothy Y. James, Prayook Srivilai, Ursula Kües, and Rytas Vilgalys

Published in: James T.Y., **Srivilai P.,** Kües U., Vilgalys, R. Evolution of the bipolar mating system of the mushroom *Coprinellus disseminatus* from its tetrapolar ancestors involves loss of mating-type-specific pheromone receptor function. Genetics **172:** 1877-1891.

This work is a cooperation between the group of Prof. Dr. Rytas Vilgalys at the Department of Biology, University of Duke and our research group Molecular Wood Biotechnology at the Institute of Forestry Botany, Georg-August-University of Göttingen. Most of this work was preformed by Dr. Timothy Y. James in frame of his Ph.D thesis who also prepared most of the manuscript. To this study, contributed the part "Expression of *Coprinellus disseminatus* genes in *Coprinopsis cinerea*" with mating type genes cloned by Dr. T. Y. James and transformed them into *C. cinerea* monokaryon 218 in order to test their functions in sexual development in a heterologous species. Transformants were further crossed to *C. cinerea* monokaryon PS004-2 to study effects in fruiting development.

7.1. Abstract

Mating incompatibility in mushroom fungi is controlled by the mating-type loci. In tetrapolar species, two unlinked mating-type loci exist (*A* and *B*), whereas in bipolar species there is only one locus. The *A* and *B* mating-type loci encode homeodomain transcription factors and pheromones and pheromone receptors, respectively. Most mushroom species have a tetrapolar mating system, but numerous transitions to bipolar mating systems have occurred. Here we determined the genes controlling mating-type in the bipolar mushroom *Coprinellus disseminatus*. Through positional cloning and degenerate PCR, we sequenced both the transcription factor and pheromone receptor mating-type gene homologues from *C. disseminatus*. Only the transcription factor genes segregate with mating-type, discounting the hypothesis of genetic linkage between the *A* and *B* mating-type loci as the causal origin of bipolar mating behavior. The mating-type locus of *C. disseminatus* is similar to the *A* mating-type locus of the model species *Coprinopsis cinerea* and encodes two tightly linked pairs of homeodomain transcription factor genes. When transformed into *C. cinerea*, the *C. disseminatus A* and *B* homologues elicited sexual reactions like native mating-type genes. Although mating-type in *C. disseminatus* is controlled by only the transcription factor genes, cellular functions appear to be conserved for both groups of genes.

7.2. Introduction

Mating in fungi is controlled by the loci that determine the mating-type of an individual, and only individuals with differing mating-types can mate. Basidiomycete fungi have evolved a unique mating system, termed tetrapolar or bifactorial incompatibility, in which mating-type is determined by two unlinked loci; compatibility at both loci is required for mating to occur. The origin of the tetrapolar mating system in the basidiomycetes is likely to be ancient since it is observed in at least two of the three major lineages, the Ustilaginomycetes, or smut fungi, and the Hymenomycetes, primarily the mushroom fungi (Burnett 1975). Also unique to the basidiomycetes is the presence of multiple alleles at the mating-type loci that allows most individuals within a population to be mating-compatible with one another. Only the mushroom-forming homobasidiomycetes possess a large allelic series at both loci, typically termed the *A* and *B* mating-type loci (Whitehouse 1949; Raper 1966).

The multiallelic tetrapolar mating system is considered to be a novel innovation that could have only evolved once (Raper 1966; Raper and Flexer 1971). For this reason, the ancestor of the homobasidiomycetes is accepted having a tetrapolar mating system. Although most (~65%) of the homobasidiomycetes possess a tetrapolar mating system, many species (~25%) instead have a bipolar system controlled by a single locus with multiple alleles (Raper 1966). The distribution of bipolar species is scattered throughout the homobasidiomycete phylogeny, suggesting multiple independent origins (Hibbett and Donoghue 2001). The population genetic consequence of the bipolar versus the tetrapolar mating system is a difference in the amount of interbreeding permitted between the haploid progeny from a single parent (siblings). Specifically, the potential for inbreeding is higher in the bipolar system because 50% of full-sib progeny are mating compatible, whereas only 25% are in the tetrapolar case. In matings between unrelated individuals, however, the frequencies of compatible mating are similar between the two mating systems; both are extremely high due to the large number of mating-type alleles (Stamberg and Koltin 1973).

Using the model tetrapolar species *Schizophyllum commune* and *Coprinopsis cinerea* (=*Coprinus cinereus*), the *A* mating-type loci of both species and the *B* mating type locus of *S. commune* were discovered to be comprised of two tightly linked subloci, the and subunits (Raper et al. 1960; Day 1960). Each unique combination of alleles at the subloci specifies a unique mating-type, making the subloci redundant in function. Less information exists on the mating-type loci of bipolar species. One puzzling finding was that attempts to dissect the bipolar mating-type locus into component subloci failed, suggesting a different genetic architecture of the bipolar locus (Raper 1966).

The frequent evolution of bipolar species suggests that the transition from a tetrapolar mating system to bipolar may have a simple genetic basis. One clue to the genetic mechanism is the absence of documented reversals from a bipolar system back to a tetrapolar one. Raper (1966) put forward three plausible hypotheses concerning the origin of the bipolar mating system. One hypothesis is based on the observation that primary mutations at either of the mating-type loci often display a self-compatible phenotype, resulting in bipolar mating behavior of normally tetrapolar strains possessing such mutated alleles. Such mutants have been recovered many times both by spontaneous origin and mutagenesis studies (Raper 1966). If such self-compatible mating-type alleles reach fixation frequency in a tetrapolar population, the population would be rendered effectively bipolar. A second hypothesis concerns the potential translocation of a chromosomal segment containing one of the mating-type loci into close genetic linkage with the other, leading ultimately to fusion of the two mating-type loci into one nonrecombining region. The suggestion that the bipolar mating-type locus is a single, indivisible locus gives credence to this hypothesis. A final hypothesis is that the function of one of the mating-type loci could be gradually assumed by the other locus. This hypothesis relates to the broadly applicable finding that the *A* and *B* mating-type loci control distinct but interconnected roles in the process of sexual development of fruiting in mushrooms (Raper 1978).

Although Raper's hypotheses were formulated before any fungal mating-type genes had been cloned, they are equally plausible today. Detailed molecular investigation of the mating-type genes of basidiomycetes has demonstrated that the mating-type genes of the smut fungi and Hymenomycetes are homologous (Hiscock and Kües 1999; Casselton and Olesnicky 1998). The *A* mating-type locus encodes for one or more pairs of homeodomain transcription factors. Each pair is comprised of two classes of homeodomain transcription factor proteins, the HD1 and HD2 proteins, which share similarity with the mating-type proteins of *Saccharomyces cerevisiae* (Hiscock and Kües 1999). Heteroallelic but not homoallelic HD1 and HD2 proteins can heterodimerize, creating a transcription unit capable of initiating the *A* mating-type specific developmental sequence (Kües and Casselton 1992). The *B* mating-type locus of the basidiomycetes was shown to encode both small peptide pheromones and seven-pass transmembrane receptors that are believed to be coupled to a trimeric G-protein complex (Brown and Casselton 2001). As with the *A* locus, pheromones can only activate heteroallelic *B* locus receptors.

Knowledge regarding the molecular sequence, organization, and function of the mating-type genes allows a reassessment of the manner in which a bipolar mating system might evolve from a tetrapolar one. The nature of the self-compatible mutant mating-types of *C. cinerea* and *S. commune* has been investigated by DNA sequencing (Olesnicky et al. 1999; Olesnicky et al. 2000; Fowler et al. 2001). Self-compatible mutants of the *B* mating-type of *C. cinerea* were created by single amino

acid substitutions in the pheromone receptors that caused either illegitimate interactions with homoallelic pheromone or constitutive activation of the *B* pathway (Olesnicky et al. 1999; Olesnicky et al. 2000). For the *A* mating-type locus of *C. cinerea*, two primary mutations causing self-compatible phenotypes were investigated and found to be the result of a deletion/recombination event that caused the inframe fusion of HD1 and HD2 genes from the same *A* haplotype but from different subloci (Kües et al. 1994a; Pardo et al. 1996). Thus, self-compatible mating-types may arise through mutation and provide a simple explanation for the origin of bipolar mating behavior through loss of discrimination by one of the two mating-type loci of a tetrapolar species. However, there is no evidence that self-compatible mating-types are involved in the origin of bipolar mating systems in the basidiomycetes, nor is there any evidence of such alleles in natural populations.

The origin of a bipolar mating system from a tetrapolar one has been addressed only in the smut fungi. In a landmark study, Bakkeren and Kronstad (1994) demonstrated that the bipolar mating-type locus of *Ustilago hordei* was formed from the fusion of the *a* and *b* mating-type loci observed in tetrapolar smut fungi into one nonrecombining mating-type region with two alleles. Thus, genetic linkage through translocation is the best explanation for the origin of bipolar mating in *U. hordei*, conforming to one of Raper's postulated mechanisms.

Recently, the bipolar mating system of the mushroom *Pholiota nameko* was characterized using linkage mapping and DNA sequencing (Aimi et al. 2005). These data demonstrated linkage between the mating-type locus and an *A* mating-type homologue but not a *B* mating-type homologue. Though intriguing, these results fail to differentiate among the possible genetic mechanisms of evolving a bipolar mating system, particularly because multiple *B* mating-type homologues exist in most mushroom genomes (this study), and only one *B* homologue was investigated in *P. nameko*.

The mushrooms in the genus *Coprinus* (*sensu lato*) provide an excellent group with which to study the evolution of mating systems and mating-type genes, because every known mating system is represented by multiple species. Furthermore, the molecular control of mating-type in mushrooms has been intensely studied in *C. cinerea*, simplifying cloning and comparative analyses. In this paper, we investigated the genetic architecture of the bipolar mating system of the common wood-decaying fungus *Coprinellus* (=*Coprinus*) *disseminatus*. Using knowledge of the mating-type loci in other homobasidiomycetes, we cloned and sequenced DNA regions containing homologues of the mating-type genes. We then used a population genetic approach to determine what changes in the mating-type genes of the hypothetical tetrapolar ancestor might have occurred during its transition to a bipolar system.

7.3. Materials and methods

7.3.1. Study species

Coprinellus disseminatus (Pers. ex Fr.) J. E. Lange is a common mushroom species that fruits in large troops on stumps, buried wood, tree tip-up mounds, and logs (Buller 1924). Its distribution is probably cosmopolitan, but appears to be divided into at least three divergent phylogenetic groups based on ribosomal DNA sequencing (Ko et al. 2001). The dikaryotic mycelium contains sparse but large clamp-connections (Lange 1952; Butler 1981). The mating system was determined by Lange (1952) and judged to be bipolar, confirming earlier results by Vandendries and Quintanilha (cited in Lange 1952). Lastly, Lange (1952) found one collection from New Delhi, India that appeared to be intersterile with seven European collections, suggesting the presence of multiple biological species within the morphological species.

7.3.2. Culture isolation and growth

Homokaryotic strains were derived from wild collected fruiting bodies, collected in 2000-2001 from within a ~15 km radius of the Duke University Campus in Durham, NC (Table 1). One fruiting body was collected per stump or mound. After fruiting bodies were collected in the field or greenhouse, they were placed over aluminum foil for at least 1 hour to collect the dark brown spores. Spores were scraped from the foil into H_2O, and a series of dilutions were plated on half-strength Emerson's YpSS (Y/2) nutrient agar plates with 1.5% agar (Stevens 1974). Following 1-2 days of growth at RT, hyphal colonies were inspected under the microscope at 100X to verify they derived from a single spore. Single spore isolates were subcultured at least twice on Y/2. Long term storage was on 1.5% malt extract agar slants at 4º. Also included were three homokaryotic European strains and a Japanese dikaryotic strain (IFO 30972). The Japanese strain was fruited in the greenhouse on a substrate composed of sawdust, rye berries, and soil in a 4:1:1 ratio. Two mating compatible homokaryons from this dikaryotic strain were isolated for further study.

7.3.3. Mating compatibility tests

Single spore isolates were obtained (n=5-14) for each of the 24 wild collected fruiting bodies. Each of these F1 progeny arrays were intra-crossed in all possible combinations to identify suitable testers representing the two mating-type alleles of the F0 fruit body. Using the tester strains, all homokaryotic strains (n=51) were paired in a complete cross design to determine if any mating-types were repeated in the population sample. All crosses were conducted on 10 cm Y/2 agar plates by inoculation of the two strains in the center of the plate ~1 cm from each other. Plates

were incubated in the dark at RT for 1-2 weeks. The formation of the dikaryon in a genetic cross typically results in vigorous growth from the margins of the paired homokaryons. Large clamp connections become apparent, albeit sparse, at cell junctions at 100X magnification. Finally, after ~2 weeks, brownish rhizomorphic strands clearly distinguish pairs that have mated. We verified dikaryotization based on the production of clamp connections in all crosses.

7.3.4. DNA amplification and sequencing

Mycelium for DNA extraction was prepared by stationary growth of isolates in 2 ml of 1.5% malt extract broth until stationary phase. The mycelium was removed from the broth, rinsed in H_2O, and lyophilized. Approximately 50 mg were used for DNA extraction using a CTAB buffer following Zolan and Pukkila (1986).

In order to study homologues of the putative *B* mating-type genes, we used degenerate PCR primers to amplify *STE3*-like pheromone receptors from homokaryotic strains following standard PCR protocols (James et al. 2004a). Primers br1-F and br1-1R were used to amplify two receptors each from C345.1 and TJ01/19.2; in addition, we used primers br2-F and br2-2R to amplify a small *STE3*-like fragment from strain TJ01/19.2 (see James et al. 2004b for primer sequences). All degenerate PCR amplicons were gel purified using a QIAquick Gel Extraction Kit (Qiagen), ligated into pCR2.1 (Invitrogen), and transformed into *Escherichia coli* strain TOP10 (Invitrogen). Plasmid templates for DNA sequencing were prepared using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced on both strands using universal forward and reverse M13 primers. Sequencing reactions utilized the BigDye sequencing kit (Applied Biosystems) and were analyzed on an ABI3700 DNA sequencer.

We screened our population sample for DNA polymorphisms at gene regions both linked and unlinked to the mating-type locus using the non-degenerate PCR primers shown in Table 2. All reactions (except *Aα* and *Aβ*) were conducted in a similar manner to that used for degenerate PCR with annealing temperatures fixed at 50ºC. Amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced as above.

7.3.5. Cosegregation analyses

We previously studied the cosegregation of *MIP* and mating-type among 13 single spore progeny of field collection TJ00/38 (James et al. 2004a). This same progeny array was used to determine whether the *STE3*-like pheromone receptors (*CDSTE3.1*, *CDSTE3.2*, and *CDSTE3.3*) also cosegregate with mating-type. Amplification of the genes was accomplished with the PCR primers shown in Table 2. Amplicons were digested using the enzymes *Sin*I for *CDSTE3.1*, *Bam*HI

for *CDSTE3.2*, and *Mwo*I for *CDSTE3.3*, following the manufacturer's instructions (New England Biolabs; Promega). Digested amplicons were electrophoresed on 1-2% agarose gels and scored for the polymorphic restriction fragments.

7.3.6. Long distance PCR and amplicon sequencing

We used long distance PCR to amplify *Aα* and *Aβ* subloci from homokaryons using the enzyme *LA Taq* (Takara) following the manufacturer's instructions. The primers for amplification are given in Table 2. The thermocycling parameters used were: initial denaturation at 94ºC for 1 min., followed by 35 cycles of 94º for 30 sec 60º for 30 sec, and 72º for 4 min, and lastly, a 10 min extension at 72º. Amplicons were digested the enzyme *Msp*I (Promega), and a subset (8 of *Aα* and 3 of *Aβ*) were chosen for DNA sequencing. Amplicons were purified using the QIAquick Gel Extraction Kit and ligated into the vector pCR2.1-TOPO (Invitrogen). The resulting plasmids were sequenced using a combination of standard subcloning procedures using plasmid pUC119 (Sambrook et al. 1989) and the GeneJumper kit containing kanamycin or chloramphenicol resistance transposons (Invitrogen). Plasmid sequencing from both ends of the transposon used the primers GJSeq-A3 and GJSeq-B2 for kanamycin and GJSeq-A3 and GJSeq-B4 for chloramphenicol (James et al. 2004b).

7.3.7. Cosmid library construction, screening, and sequencing

A cosmid library was prepared in the vector SuperCos-Pab1 (Bottoli et al. 1999) using the DNA of strain C345.1. This strain has been deposited into the Belgian Co-ordinated Collections of Micro-organisms as MUCL 43037. DNA of C345.1 was prepared from mycelium grown in 1 L of Y/2 broth under rotary shaking (~125 rpm) at RT. Preparation and screening of the library followed the protocol of James et al. (2004b). The library was screened by PCR of bacterial cells for two genes, *MIP* and *CDSTE3.1* (see Table 2 for primer sequences). DNA of all cosmid clones was isolated using the QIAprep Spin Miniprep Kit (Qiagen). Six overlapping cosmids were sequenced for the *MIP*/mating-type locus region using a random shotgun subcloning method involving partial digestion with restriction enzymes (Zhou et al. 1988). A single *CDSTE3.1* positive cosmid, C25.B2.5, was also sequenced using the GeneJumper primer insertion kit with the kanamycin resistance transposon (Invitrogen). Sequencing reactions were accomplished using the BigDye kit and primers GJSeq-A3 and GJSeq-B2. Assembly of sequences traces into contigs was performed with Sequencher v4.1 (Gene Codes). Gaps in the contigs were filled by primer walking with synthesized oligonucleotides (Operon). Approximately 4.0-fold coverage of the 75.5 kb *MIP* chromosomal region and 3.1-fold coverage of the 41.9 kb *CDSTE3.1* cosmid were achieved.

7.3.8. Expression of *C. disseminatus* genes in *C. cinerea*

C. cinerea monokaryon 218 (*A3*, *B1*, *trp1.1,16, bad*; Binninger et al. 1987; Kües et al. 2002) was transformed according to the protocol given by Granado et al. (1997). The *trp1*$^{+}$ vector pCc1001 (Binninger et al. 1987) was used in co-transformations with plasmid pCR2.1-TOPO containing the entire *Aα* sublocus from strains TJ00/99.1 and TJ01/16.3 (plasmids 99.1A and 16.3A) and the entire *Aβ* sublocus from strains TJ00/99.1 and TJ00/89.2 (99.1A and 89.2A). Also used in co-transformation with pCc1001 were C25.B2.5 containing the two putative pheromone receptor genes *CDSTE3.1* and *CDSTE3.3* and three pheromone genes *CDPHB1*, *CDPHB2.1* and *CDPHB2.2*, and C25_e1.10, a 10 kb subclone of C25.B2.5 containing *CDSTE3.1* and *CDPHB1* in vector pZERO (Invitrogen). Co-transformations used 1 µg DNA for each plasmid. Transformants were picked onto minimal medium (Granado et al. 1997) and checked for *A*-regulated clamp cell production (Kües et al. 1992) and *B*-regulated subapical peg formation and clamp cell fusion (Badalyan et al. 2004) under a microscope. Functional expression of pheromone receptor and/or pheromone genes was further analyzed in mating reactions (O´Shea et al. 1998) on YMG/T medium (Granado et al. 1997) with monokaryon PS004-2 (*A42*, *B1*; P. Srivilai, unpublished). Fruiting abilities of dikaryons were tested under *C. cinerea* standard fruiting conditions (Granado et al. 1997). A minimum of 20 transformants were analyzed per co-transformation experiment. Control transformations utilized solely pCc1001.

Table 1 Geographic origin and mating-type of homokaryotic strains of *C. disseminatus* used in the population survey

Strain	Mating-type	Origin
C345.1	*A1*	France
TJ00/38.3	*A2*	Duke Forest, Korstian Division, North Carolina
TJ00/38.6	*A3*	Duke Forest, Korstian Division, North Carolina
TJ00/89.1	*A4*	Duke Forest, Durham Division, North Carolina
TJ00/89.2	*A3*	Duke Forest, Durham Division, North Carolina
TJ00/91.1	*A5*	Near Northgate Park, Durham, North Carolina
TJ00/91.2	*A6*	Near Northgate Park, Durham, North Carolina
TJ00/94.4	*A7*	Duke Forest, Durham Division, North Carolina
TJ00/94.5	*A8*	Duke Forest, Durham Division, North Carolina
TJ00/99.1	*A5*	Duke University Campus, North Carolina
TJ00/99.3	*A9*	Duke University Campus, North Carolina
TJ00/100.1	*A10*	Innsbrook, Austria
TJ00/100.3	*A11*	Innsbrook, Austria
TJ01/02.5	*A12*	Duke Forest, Durham Division, North Carolina
TJ01/02.7	*A5*	Duke Forest, Durham Division, North Carolina
TJ01/04.1	*A13*	Duke University Campus, North Carolina
TJ01/04.4	*A14*	Duke University Campus, North Carolina

Table 1 (continued)

TJ01/05.1	*A15*	Duke Forest, Blackwood Division, North Carolina
TJ01/05.8	*A16*	Duke Forest, Blackwood Division, North Carolina
TJ01/06.2	*A17*	Duke Forest, Blackwood Division, North Carolina
TJ01/06.5	*A18*	Duke Forest, Blackwood Division, North Carolina
TJ01/07.1	*A19*	Duke Forest, Blackwood Division, North Carolina
TJ01/07.6	*A20*	Duke Forest, Blackwood Division, North Carolina
TJ01/08.1	*A21*	Duke Forest, Blackwood Division, North Carolina
TJ01/08.2	*A22*	Duke Forest, Blackwood Division, North Carolina
TJ01/09.1	*A23*	Duke Forest, Blackwood Division, North Carolina
TJ01/09.3	*A24*	Duke Forest, Blackwood Division, North Carolina
TJ01/10.2	*A4*	Duke Forest, Blackwood Division, North Carolina
TJ01/10.3	*A25*	Duke Forest, Blackwood Division, North Carolina
TJ01/11.2	*A26*	Duke Forest, Blackwood Division, North Carolina
TJ01/11.5	*A27*	Duke Forest, Blackwood Division, North Carolina
TJ01/12.4	*A28*	Duke Forest, Blackwood Division, North Carolina
TJ01/12.6	*A29*	Duke Forest, Blackwood Division, North Carolina
TJ01/13.3	*A30*	Duke Forest, Blackwood Division, North Carolina
TJ01/13.8	*A31*	Duke Forest, Blackwood Division, North Carolina
TJ01/14.1	*A32*	Duke Forest, Korstian Division, North Carolina
TJ01/14.2	*A21*	Duke Forest, Korstian Division, North Carolina
TJ01/15.1	*A33*	Duke Forest, Korstian Division, North Carolina
TJ01/15.4	*A34*	Duke Forest, Korstian Division, North Carolina
TJ01/16.1	*A35*	Duke Forest, Korstian Division, North Carolina
TJ01/16.3	*A36*	Duke Forest, Korstian Division, North Carolina
TJ01/17.1	*A26*	Duke Forest, Korstian Division, North Carolina
TJ01/17.6	*A37*	Duke Forest, Korstian Division, North Carolina
TJ01/18.1	*A38*	Duke Forest, Korstian Division, North Carolina
TJ01/18.5	*A39*	Duke Forest, Korstian Division, North Carolina
TJ01/19.2	*A31*	Duke Forest, Durham Division, North Carolina
TJ01/19.4	*A40*	Duke Forest, Durham Division, North Carolina
TJ01/20.5	*A41*	Duke University Campus, North Carolina
TJ01/20.6	*A13*	Duke University Campus, North Carolina
IFO 30972.16	N. D.[a]	Japan
IFO 30972.17	N. D.	Japan

[a] Homokaryons isolated from dikaryotic strain IFO 30972 appear to be intersterile with European and North American isolates.

Supplementary Table 1 Genes identified in the region surrounding the *C. disseminatus* mating-type locus. Results are from BLASTX homology searches against the NCBI database. P-value indicates the probability of the match being due to chance in GenBank similarity searches. Listed under homologue is the BLAST hit with the lowest P-value that also has been assigned a functional class. The species from which the lowest P-value hit was obtained is given in brackets, followed by the GenBank accession number in parentheses

Gene	P-value	Position	Homologue	Possible function
PAB1	0.0	(282-2,642)	para-aminobenzoic acid synthetase [*Coprinus cinereus*] (AAF89583)	Amino acid/vitamin metabolism
CDUP1	$1x10^{-140}$	(4,146-2,779)	hypothetical protein [*Neurospora crassa*] (XP_327825)	isopenicillin N synthase and related dioxygenases
UBC12	$3x10^{-46}$	(4,446-5,274)	probable E2 ubiquitin-conjugating enzyme [*Neurospora crassa*] (CAD21285)	proteasome-mediated degradation
CDUP2	$1x10^{-12}$	(6,082-5,539)	COG2351: Transthyretin-like protein [*Pseudomonas fluorescens* PfO-1] (ZP_00262846)	hormone transport
CDUP3	$3x10^{-24}$	(6,263-7,429)	NNP-1 protein (Novel nuclear protein 1) (Nucleolar protein Nop52 [*Homo sapiens*] (P56182)	ribosomal RNA processing
CDUP4	$1x10^{-4}$	(8,355-9,162)	hypothetical protein [*Yarrowia lipolytica*] (XP_505090)	unknown
CDUP5	$2x10^{-24}$	(10,051-11,934)	hypothetical protein CNBB5120 [*Cryptococcus neoformans* var. *neoformans* B-3501A] (EAL22337)	unknown
CDUP6	$8x10^{-10}$	(13,251-14,509)	hypothetical protein B24M22.170 [*Neurospora crassa*] (T51221)	unknown
GLYDH	0.0	(14,970-18,118)	glycine dehydrogenase [decarboxylating], mitochondrial precursor [*Pisum sativum*] (P26969)	glycine metabolism
CDUP7	$3x10^{-94}$	(20,327-18,387)	hypothetical protein PDUPA1 [*Pleurotus djamor*] (AAS46735)	unknown
SEC61	$2x10^{-6}$	(20,946-21,279)	Sec61, gamma subunit [*Mus musculus*] (NP_035473)	intracellular trafficking and secretion
CDUP8	$1x10^{-14}$	(27,599-25,683)	hypothetical protein CNBC0460 [*Cryptococcus neoformans* var. *neoformans* B-3501A] (EAL21905)	unknown

Supplementary Table 1 (continued)

CDUP9	$3x10^{-63}$	(29,224-27,913)	hypothetical protein PDUPA2 [*Pleurotus djamor*] (AAS46739)	unknown
CDUP10	$3x10^{-13}$	(29,410-30,163)	hypothetical protein CNBF3430 [*Cryptococcus neoformans* var. *neoformans* B-3501A] (EAL20016)	unknown
CDUP11	$3x10^{-11}$	(30,306-31,837)	hypothetical protein CNBD1900 [*Cryptococcus neoformans* var. *neoformans* B-3501A] (EAL21496)	unknown
MIP	0.0	(32,899-35,388)	Mitochondrial intermediate peptidase, mitochondrial precursor [*Schizophyllum commune*] (P37932)	cleavage of mitochondrial precursor proteins
CDA1	$1x10^{-41}$	(37,979-35,896)	mating type protein [*Coprinus cinereus*] (AAA59366)	mating-type specific homeodomain transcription factor (HD1)
CDA2	$2x10^{-29}$	(38,312-39,865)	homeodomain type 2 mating protein a2-1 [*Coprinus bilanatus*] (AAK17070)	mating-type specific homeodomain transcription factor (HD2)
CDB1	$5x10^{-36}$	(43,501-41,459)	mating-type protein beta 1 [*Coprinus cinereus*] (AAD33325)	mating-type specific homeodomain transcription factor (HD1)
CDB2	$2x10^{-31}$	(43,740-45,534)	*A* mating type protein – inky cap [*Coprinus cinereus*] (S47143)	mating-type specific homeodomain transcription factor (HD2)
ß-FG	$4x10^{-30}$	(46,725-45,784)	hypothetical protein CNBJ3430 [*Cryptococcus neoformans* var. *neoformans* B-3501A] (EAL18420)	unknown
GLGEN	$2x10^{-40}$	(51,817-48,642)	glycogenin [*Neurospora crassa*] (AAS68518)	lipopolysaccharide biosynthesis and glycogen synthesis
YPL109	0.0	(51,945-54,052)	hypothetical protein PDUPA3 (similar to *S. cerevisiae* YPL109) [*Pleurotus djamor*] (AAS46740)	predicted unusual protein kinase
BLAC1	$3x10^{-15}$	(56,319-54,207)	hypothetical protein MG08486.4 [*Magnaporthe grisea* 70-15] (EAA49571)	Beta-lactamase class C and other penicillin binding proteins
BLAC2	$3x10^{-18}$	(59,318-57,398)	hypothetical protein FG08136.1 [*Gibberella zeae* PH-1] (EAA71935)	Beta-lactamase class C and other penicillin binding proteins
MAD2	$6x10^{-12}$	(59,953-60,707)	mad2-like protein; possible mitotic checkpoint [*Schizosaccharomyces pombe*] (NP_595951)	spindle checkpoint protein

Supplementary Table 1 (continued)

SNX4	$4x10^{-15}$	(63,071-61,061)	putative nexin sorting protein (similarity to yeast snx4) [*Schizosaccharomyces pombe*] (NP_593905)	intracellular trafficking and secretion
CDUP12	$2x10^{-17}$	(63,127-65,495)	putative mannosyltransferase [*Schizosaccharomyces pombe*] (CAB16193)	putative mannosyltransferase
RPB2	0.0	(69,665-65,635)	DNA-dependent RNA polymerase II second largest subunit [*Amanita phalloides*] (AAS67498)	RNA polymerase
CDUP13	$3x10^{-8}$	(71,228-70,061)	Fructosamine-3-kinase [*Nostoc punctiforme*] (ZP_00106292)	possible fructosamine-3-kinase
QDR1	$1x10^{-25}$	(73,482-71,368)	quinidine resistance; Qdr1p [*Saccharomyces cerevisiae*] (NP_012146)	carbohydrate transport and metabolism
NDSE	$8x10^{-31}$	(74,876-73,653)	Predicted nucleoside-diphosphate-sugar epimerases [*Magnetospirillum magnetotacticum*] (ZP_00054963)	Cell envelope biogenesis, outer membrane/ Carbohydrate transport and metabolism

Table 2 PCR primers used to survey genetic variation in *C. disseminatus*

Gene	**Primer**	**Sequence 5′→ 3′**
PAB1	CdPAB1-F	CGTACGACTCATTCACACACAA
	CdPAB1-R	ACGGACTCTGGGTGGTACTG
GLYDH	CdGLYDH-F	TATCGACTGGCACTGCAAAG
	CdGLYDH-R	CAACTACCGAGGGCAATCAT
MIP	CdMIP-F	CTGCGGGCAACTGGRAACAA
	CdMIP-R	GAAGGACGTCTCTGGCACATA
CDHH	CdHH-F	ACTGTGGAGGCAAGTCGAAG
	CdHH-R	CAAACTTCTGCCACTCAGCA
CDRF	CdRF-F	GTTCCCATCCCAACAAACTG
	CdRF-R	AATAGGAACGCGTCTGAGGA
YPL109	CdYPL109-F	AGGCACCTTTGAGCCTCTCT
	CdYPL109-R	ACCCATACCCACACCTTCAA
RPB2	CdRPB2-F	AGCCGACGGAGATACATGAC
	CdRPB2-R	CGTATTCGTTACGCACAGGA
CDSTE3.1	CdSTE3.1-F	CATCGCTCCTGTATGGTGTG
	CdSTE3.1-R	CTGGAGAATAGGGACGCAAA
CDSTE3.2	CdSTE3.2-F	TCGATCGTATGGAACGRTAA
	CdSTE3.2-R	AKCGTCTAGGYGTGAGGTTC
CDSTE3.3	CdSTE3.3-F	CCCATTTGGTGTGACATCTG
	CdSTE3.3-R	GGTCAAGAGCTGGCTGAACT
CDPHB1	CdPHB1-F	TACCGAAGAATCAGGCCTCT
	CdPHB1-R	CGATGTTACAGAACGCACCA
Aα	Cd -F	GGCGTATATCAGCTGCCACT
	Cd -R	CCCYTCCTTTCGATCTTTTC
Aβ	Cd -F	AGGCCGAAAAGATCGAAAGG
	Cd -R	GGCACGGAGAAGATTTYACTGG

Supplementary Table 2 Genes identified from the chromosomal region surrounding the *CDSTE3.1* gene. Identifications are based on BLASTX searches to the NCBI database (Altschul et al. 1997). P-value indicates the probability of a false match. Homologue indicates the best matching protein assigned to a functional class, with the organism in brackets and accession number in parentheses. N. S. indicates a peptide pheromone that is predicted, but shows no significant match in BLAST searches

Gene	P-value	Position	Homologue	Possible function
RAB7.1	$6x10^{-28}$	(1,946-1,305)	putative GTP-binding protein [*Cucumis sativus*] (AAQ72787)	GTPase implicated in vesicle trafficking
RAB7.2	$2x10^{-57}$	(4,378-3,399)	RAB small monomeric GTPase, putative [*Cryptococcus neoformans* var. *neoformans* JEC21] (AAW46112)	GTPase implicated in vesicle trafficking
CDUP14	$4x10^{-58}$	(7,377-5,787)	hypothetical protein PDUPB2 [*Pleurotus djamor*] (AAS46753)	possible transcription factor
ERG26	$4x10^{-43}$	(8,099-9,435)	C-3 sterol dehydrogenase [*Cryptococcus neoformans* var. *grubii* H99] (AAQ88129)	ergosterol biosynthesis
CDSTE3.1	$4x10^{-74}$	(21,903-20,027)	pheromone receptor Rcb3 B45 [*Coprinopsis cinerea*] (AAQ96348)	G-protein-coupled transmembrane receptor
CDPHB1	N.S.	(22,492-22,704)	Phb2.1.42 [*Coprinus cinereus*] (AAF01425)*	small peptide pheromone
PERO	$2x10^{-66}$	(25,046-23,402)	peroxidase [*Coprinopsis cinerea*] (CAA49216)	oxidoreductase; lignin degradation
CDUP15	$7x10^{-29}$	(27,651-27,066)	hypothetical protein CNBF0210 [*Cryptococcus neoformans* var. *neoformans* B-3501A] (EAL20209)	unknown
CDTR	$2x10^{-11}$	(30,293-29,376)	putative tyrosine recombinase [*Danio rerio*] (AAN71722)	putative retroelement
CDRT	$9x10^{-22}$	(33,475-30,471)	polyprotein [*Danio rerio*] (DAA01994)	putative retroelement
CDPHB2.1	N. S.	(34,602-34,805)	No match	small peptide pheromone
CDSTE3.3	$1x10^{-101}$	(38,103-36,555)	pheromone receptor Rcb3 B5 [*Coprinopsis cinerea*] (AAQ96347)	G-protein-coupled transmembrane receptor
CDPHB2.2	N. S.	(39,645-39,114)	Phb1.1.42 [*Coprinus cinereus*] (AAF01421.1)*	small peptide pheromone

7.3.9. Data analyses

Identification of genes on the sequenced cosmid clones used homology searching of the GenBank database with the BLASTX algorithm (Altschul et al. 1997). CDD searches were also used to determine putative conserved domains that helped in the determination of gene function (Marchler-Bauer and Bryant 2004). Searches for the small peptide pheromone genes were attempted using NCBI's ORFfinder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) with aid from the software GeneMark v2.5 using both *Schizosaccarhomyces pombe* and *Saccharomyces cerevisiae* models (Borodovsky and McIninch 1993).

Gene products of the *Aα* and *Aβ* subloci were translated into amino acid sequences and aligned using ClustalX (Thompson et al. 1997) with default parameters and adjusted by eye. Multiple sequence alignments for all other genes were performed using the alignment editor GeneDoc (Nicholas and Nicholas 1997). A phylogeny of the STE3-like pheromone receptors was conducted by analyzing the sequences from *C. disseminatus* with other pheromone receptor sequences retrieved from GenBank. Following exclusion of ambiguously aligned regions, 158 aligned amino acids were used for phylogeny reconstruction. A maximum likelihood phylogeny was reconstructed using the software PROTML from the MOLPHY package using 1,000 heuristic searches and the JTT-F substitution matrix (Adachi and Hasegawa 1996). Support for nodes was assessed using approximate bootstrap probabilities by the RELL method (Hasegawa and Kishino 1994). Basic statistics of DNA polymorphism were calculated using the software DnaSP v3.53 (Rozas and Rozas 1999) and MEGA v3.0 (Kumar et al. 2004).

7.4. Results

7.4.1. *Coprinellus disseminatus* has a bipolar mating system with multiple alleles

We obtained single spore isolates from 25 fruit body collections and intercrossed them to determine the mating-types and mating system of the progeny. All of the 25 collections displayed a bipolar mating system with only two mating-types among all progeny of a collection. In a few instances some homokaryons among the progeny of a fruit body showed marked inability to mate with other strains. Deviations from the expected pattern of bipolar mating have already been recorded in certain isolates (Lange 1952). We note that these patterns were restricted to crosses within a progeny array and that they were, without exception, due to strains that showed an inability to mate rather than a promiscuous mating pattern. We specifically avoided these strains when we chose testers for a complete population cross.

When all homokaryotic testers were intercrossed, nearly every mating was successful. We found that the Japanese homokaryons (IFO 30972.16-17) were unable to mate with any of the European or North American isolates, suggesting the Japanese strains represent a different species or intersterility group. The complete population crossing design allowed us to assign a series of mating-types (Table 1) and to estimate the number of total alleles in the species through the observation of mating-type repeats. We use the nomenclature of Raper (1966) in referring to the sole mating-type of the bipolar *C. disseminatus* as the *A* mating-type locus. Six mating-types were each shared by two strains; one mating-type (*A5*) was recovered from three strains. Using the formula of O'Donnell and Lawrence (1984), there estimated to be ~123 mating-types in the global population with 95% confidence intervals of 73 to 254. These data suggest that *C. disseminatus* has a much larger number of mating-types than most of the bipolar species studied to date (Murphy and Miller 1997).

7.4.2. The pheromone receptors of *C. disseminatus* are not part of the mating-type locus

We developed two sets of degenerate PCR primers for amplifying the *STE3*-like pheromone receptors from homobasidiomycetes. Amplification using primers br1-F and br1-1R on genomic DNA of *C. disseminatus* strain C345.1 yielded two fragments homologous to *STE3*-like pheromone receptors in the same amplification reaction. One of them, termed *CDSTE3.1* possesses the same four introns as do most homobasidiomycete *STE3* receptor genes. The other fragment (*CDSTE3.2*) was rather unique among the known *STE3* sequences in lacking three of the four introns. Amplification of genomic DNA of strain TJ01/19.2 with primers br1-F and br1-1R also produced two *STE3*-like amplicons. One of these was very similar to *CDSTE3.2*, and the other fragment represented a third receptor paralogue, *CDSTE3.3*. Amplification of DNA from strain TJ01/19.2 using PCR primers br2-F and br2-2R also yielded a fourth paralogue, *CDSTE3.4*. Using specific primers or cosmid sequencing, we were ultimately able to recover homologues of *CDSTE3.1-CDSTE.4* from strain TJ01/19.2 and homologues of *CDSTE3.1-CDSTE3.3* from strain C345.1, demonstrating at least three receptor paralogues in the two *C. disseminatus* homokaryons tested.

We have previously used PCR to amplify and genotype a small fragment of *MIP* from *C. disseminatus*, and we demonstrated that the *MIP* gene fragment cosegregates with the mating-type locus of *C. disseminatus* using a small progeny array (James et al. 2004a). PCR primers specific to the four *STE3*-like sequences were designed to test whether these putative pheromone receptors cosegregated with the mating-type locus. The segregation of three putative pheromone receptors (*CDSTE3.1-CDSTE3.3*) was examined among the progeny array of parental dikaryon TJ00/38, and all three of these receptors displayed no apparent linkage with the mating-type locus (Table 3). Two of the loci (*CDSTE3.1* and *CDSTE3.3*) displayed complete cosegregation with each other

suggesting they are closely linked in the genome. Although segregation data for *CDSTE3.4* are lacking, one piece of evidence suggests that this gene is not mating-type specific. The PCR primers specific to this gene only amplified DNA of two sibling strains (TJ01/19.2 and TJ01/19.4). These two sibling strains also possess different mating-type alleles but have identical sequences at *CDSTE3.4*.

Table 3 Segregation of mating-type and genetic markers in a progeny array derived from field collection TJ00/38. The two genotypes at the DNA loci are arbitrarily assigned to states + and – reflecting the segregating PCR-RFLP sites

Isolate	Mating-type	*MIP*	*CDSTE3.1*	*CDSTE3.2*	*CDSTE3.3*
TJ00/38.1	*A3*	–	–	+	–
TJ00/38.6	*A3*	–	–	–	–
TJ00/38.7	*A3*	–	–	–	–
TJ00/38.11	*A3*	–	–	+	–
TJ00/38.14	*A3*	–	–	–	–
TJ00/38.15	*A3*	–	–	–	–
TJ00/38.3	*A2*	+	–	–	–
TJ00/38.4	*A2*	+	+	–	+
TJ00/38.5	*A2*	+	+	+	+
TJ00/38.9	*A2*	+	+	–	+
TJ00/38.10	*A2*	+	+	–	+
TJ00/38.12	*A2*	+	–	–	–
TJ00/38.13	*A2*	+	–	+	–

7.4.3. Structure of the mating-type locus

Having demonstrated that the single mating-type locus of *C. disseminatus* cosegregates with the *MIP* gene but not with any *STE3*-like receptors, we probed a cosmid library for the *MIP* gene under the assumption that the gene would be very tightly linked to the mating-type locus, as it is in model mushroom species (Kües et al. 2001). We obtained four unique, overlapping cosmid clones that contained the *MIP* gene from the library; an additional clone was obtained by a short chromosomal walk (c94.K3.1.22). Through subcloning and DNA sequencing of these cosmids, we assembled a restriction and gene map of the *A* mating-type locus (Fig. 1).

Immediately adjacent to *MIP* were four genes homologous to the class of homeodomain transcription factors (Fig. 1, Supplementary Table 1). The genes were arranged as divergently transcribed HD1 and HD2 pairs, as seen in other *A* mating-type loci (Casselton and Olesnicky 1998). For convenience and comparison with other mushroom mating-type loci, we refer to the two pairs as *Aα* and *Aβ* subunits of the *A* mating-type locus of *C. disseminatus*. As seen with other homobasidiomycete mating-type loci, ~32 kb upstream from the mating-type genes is the *PAB1* gene, encoding for *para*-amino benzoic acid synthase (Kües et al. 2001; James et al. 2002). The *A* mating-type locus region from *C. disseminatus* was compared with the genome sequence available for the related species *C. cinerea* (Fig. 1). These data show a very conserved gene order between the two species for nearly the entire 75 kb region with the exception of a duplicated pair of putative drug binding proteins inserted into the *C. disseminatus* region (*BLAC1* and *BLAC2*). Other genes of known function displaying conserved synteny between *C. disseminatus* and other homobasidiomycetes (James et al. 2004b; T. Y. James, unpublished data), are RNA polymerase II (*RPB2*), glycine dehydrogenase (*GLYDH*) and a putative kinase with similarity to yeast protein YPL109 (*YPL109*). In summary, we sequenced a large region of the chromosome surrounding the *MIP* gene, revealing conserved synteny of this region in comparison with model mushroom species. Importantly, we discovered putative *A* mating-type genes clustered into an ~10 kb region encoding four homedomain transcription factor genes.

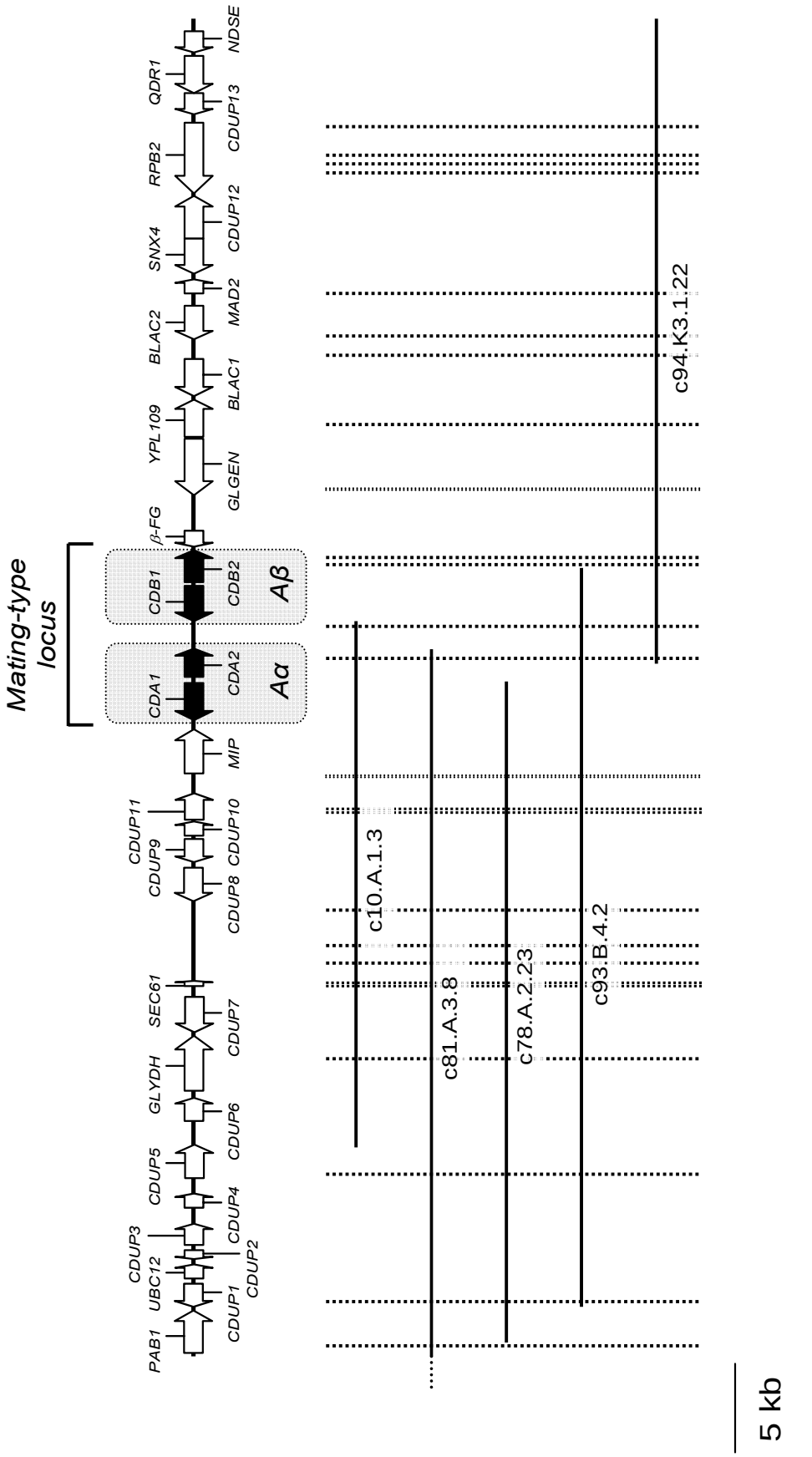


Figure 1. Restriction and gene map of the mating-type locus of *C. disseminatus*. Arrows indicate direction of transcription. Below the gene map is shown the position of the cosmids used to generate the DNA sequences. Genes were identified using BLASTX searches with a cutoff P-value of 10^{-4} (See Supplementary Table 1). Mating-type specific genes are shown as black arrows. Predicted genes with no clear cellular function are indicated as *CDUP1-CDUP13*. Vertical dashed lines connecting cosmids represent *Eco*RI cut sites. Clone c81.A.3.8 continues beyond the left border of the map.

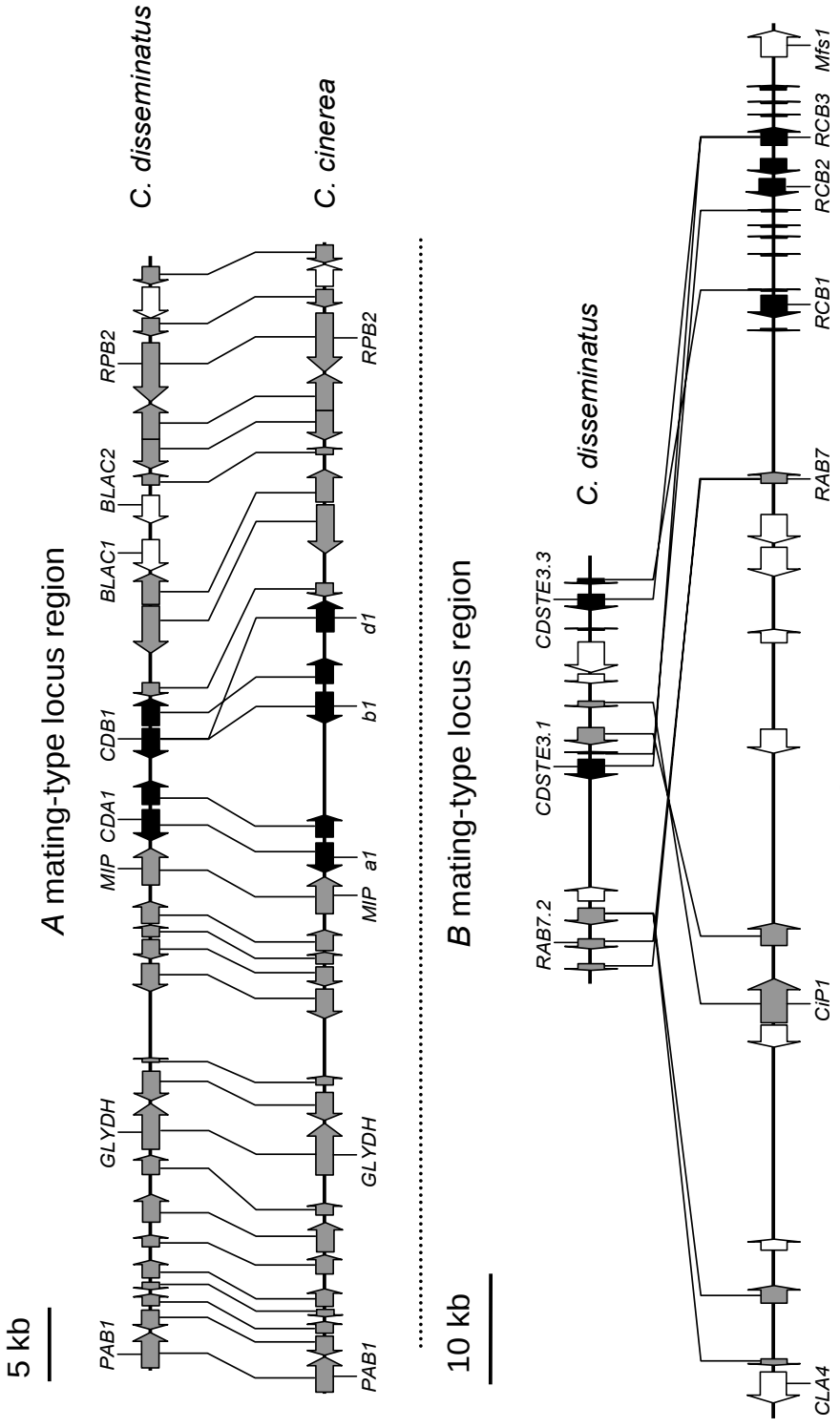


Figure 2. Schematic comparison of genomic regions from *C. cinerea* to *C. disseminatus A* and *B* mating-type loci. *C. cinerea* mating-type region *A* corresponds to 382-462 kb from accession number AACS01000026.1. *C. cinerea* mating-type region *B* corresponds to 50-176 kb from accession number AACS01000134.1. *C. disseminatus* regions are the same as shown in Figures 1 and 5. Mating-type gene homologues are shown as black arrows. Grey and white arrows indicate genes that are syntenic or not, respectively, between the two species.

7.4.4. Polymorphism at and near the mating-type locus

Basidiomycete mating-type genes have been shown to have high levels of amino acid polymorphism between alleles with substitutions clustered in the N-terminal regions of the homeodomain proteins, as this region has been determined to function in allele discrimination (Yee and Kronstad 1993; Wu et al. 1996; Banham et al. 1995; Badrane and May 1999). In order to determine the extent and location of variability of the putative *C. disseminatus* mating-type genes we used long PCR to amplify the *Aα* and *Aβ* subloci in two separate reactions. We were able to amplify almost all of the homokaryotic isolates, except the genetically distinct Japanese strains. Restriction digests of the *Aα* PCR products revealed extensive DNA diversity in *Msp*I cut sites (Fig. 3). Homokaryons with the same mating-type as determined by genetic crosses shared identical or similar restriction digest patterns, whereas for different alleles, it is difficult even to assess the homology of restriction fragments due to excess polymorphism. A similar result was found for the digests of *Aβ* amplicons (data not shown).

We cloned 8 *Aα* and 3 *Aβ* amplicons and sequenced them to determine the pattern of DNA sequence diversity in relation to the functional domains of the proteins they encode. As observed with other mating-type genes, the level of DNA sequence and amino acid diversity between alleles was tremendous. The nine *CDA1* and *CDA2* sequences could be divided into five sequence types (presumably alleles). Similarity among the five heteroalleles at the N-terminal region of the protein before the homeodomain motif ranged from 53- 69% for CDA1 and 45- 65% for CDA2. In contrast, similarity in the C-terminal region after the homeodomain motif ranged from 64-79% for CDA1 and 89-97% for CDA2.

If the genomic region surrounding the homeodomain genes in *C. disseminatus* was all part of one non-recombining region, it would be expected that same forces of balancing selection that promote sequence divergence of mating-type alleles (May et al. 1999) would also have a strong effect on increasing polymorphism of the entire region. Thus, we investigated the genetic variation the genes surrounding the *C. disseminatus* mating-type locus by sequencing six loci spaced over an ~70 kb region surrounding the mating-type locus for our sample of 49 homokaryons (non-Japanese isolates). The amount of DNA polymorphism (π) was approximately an order of magnitude higher than the genes that flank the *A* locus. Variation was significantly higher ($P < 0.05$) at the gene regions directly adjacent to the mating-type locus (i.e., *MIP*, *CDHH, CDRF*). However, the substitutions in Table 4 include both synonymous and nonsynonymous changes for protein coding genes such as *MIP*. We also looked only at the gene diversity at silent positions where substitutions have no effect on the encoded proteins (π_S), such that differences in standing genetic variation should reflect only differences in coalescence time due to balancing rather than positive selection.

The DNA diversity at silent positions was similarly low for all genes outside of the mating-type locus (Fig. 4), however, the genes upstream of the *A* locus generally had a higher level of silent polymorphism than the genes downstream of the *A* locus. These data suggest that balancing selection on the mating-type loci may have the effect of elevating polymorphism of the neighboring genes, but this effect is greatly reduced over short physical distances, presumably through recombination.

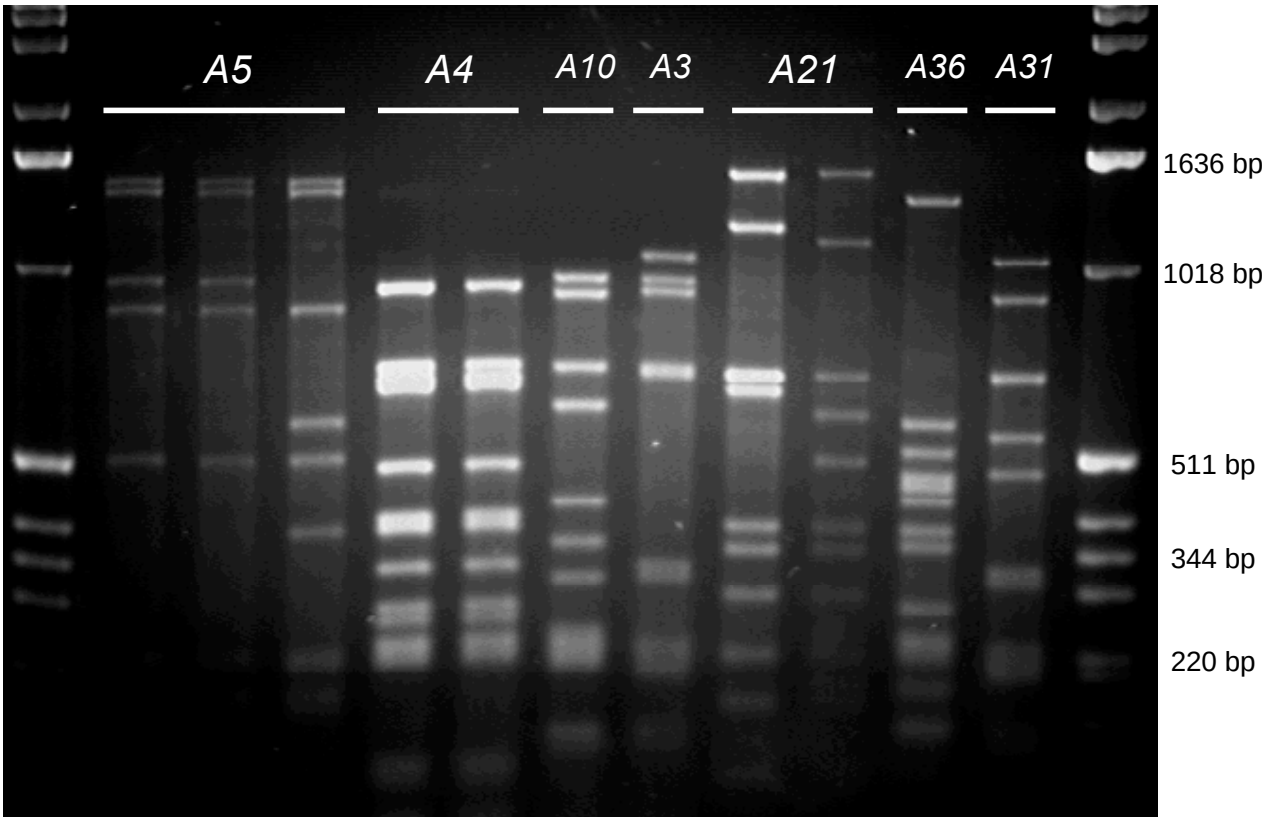


Figure 3. Restriction digests of *Aα* amplicons of *C. disseminatus* homokaryons with the enzyme *Msp*I. In lanes 1 and 13 are size standards with sizes shown to the right of the photo. Lane 2 = TJ00/91.1; lane 3 = TJ00/99.1; lane 4 = TJ01/02.7; lane 5 = TJ00/89.1; lane 6 = TJ01/10.2; lane 7 = TJ00/100.1; lane 8 = TJ00/89.2; lane 9 = TJ01/08.1; lane 10 = TJ01/14.2; lane 11 = TJ01/16.3; lane 12 = TJ01/19.2. Shown above the lanes of the digested amplicons are the mating-types of the isolates (Table 1).

7.4.5. Genetic structure of a putative extinct *B* mating-type

We probed the *C. disseminatus* cosmid library for clones containing the *CDSTE3.1* gene. The complete sequencing of one 41.9 kb *CDSTE3.1* positive cosmid clone C25.B2.5 indicated several genes in this chromosomal region, including two putative pheromone receptors and two putative small pheromone genes (Supplementary Table 2; Fig. 5). The two *STE3*-like pheromone receptor genes on this cosmid corresponded with the previously identified *CDSTE3.1* and *CDSTE3.3* genes. The predicted proteins encoded by *CDSTE3.1* and *CDSTE3.3* are 536 and 426 amino acids in length, both contain the canonical five introns observed in other homobasidiomycete pheromone receptors, and both appear to have seven transmembrane spanning helices and a long cytoplasmic tail as predicted by the program HMMTOP (Tusnády and Simon 2001). The phylogenetic origin of these receptors is discussed below.

Searches for possible pheromone genes in the *CDSTE3.1* region revealed three genes encoding putative peptide pheromones (*CDPHB1*, *CDPHB2.1*, and *CDPHB2.2*; Fig. 5). One pheromone gene is in close proximity to the receptor *CDSTE3.1*, and two are found in the genomic region surrounding *CDSTE3.3*. Only two of the three pheromone genes show a significant match with any published pheromone sequences (Supplementary Table 2), but all proteins were predicted as probable ORFs or exons using the GeneMark algorithm (Borodovsky and McIninch 1993). Olesnicky et al. (1999) suggested that the conserved ER motif found 11 amino acids N-terminal to the modified cysteine of pheromone protein phb2.2 of *C. cinereus* was likely to be the cleavage site to release the mature peptide. All three putative *C. disseminatus* pheromones display this pair of amino acids in position similar to *C. cinereus* phb2.2 and other homobasidiomycete pheromones (Fowler et al. 2001; Riquelme et al. 2005).

The region of the *C. disseminatus* genome containing the two pheromone receptors demonstrates some conserved gene order with the *B* mating-type locus of *C. cinerea* (Fig. 2). The gene regions from the two species share four genes (*PERO*, *RAB7*, *CDUP14*, and *CDUP15*) and the pheromone and pheromone receptor genes. However, the amount of gene rearrangement at the *B* mating-type locus is very high compared to the synteny of the *A* mating-type loci of *C. cinerea* and *C. disseminatus* (Fig. 2). Genes with known function from the *CDSTE3.1* region include *ERG26*, encoding a putative dehydrogenase involved in ergosterol biosynthesis, *PERO*, encoding a protein with strong similarity to lignin degrading peroxidases, and two *RAB7* genes, encoding putative GTPases involved in vesicle trafficking.

Table 4 DNA diversity at *C. disseminatus* loci. n = number of monokaryotic samples sequenced; N = number of aligned basepairs; S = no. of segregating sites; = nucleotide diversity or the average number of pairwise differences per site (using Jukes-Cantor correction). Nucleotide diversities are followed by standard errors in parentheses estimated using 500 bootstrap replicates (Kumar et al. 2004)

Gene	n	N	S	π
CDA1 **(exons)**	9	1863	1024	0.371 (0.010)
CDA2 **(exons)**	9	1524	213	0.222 (0.009)
CDB1 **(exons)**	4	1941	948	0.475 (0.015)
CDB2 **(exons)**	4	1668	920	0.565 (0.016)
PAB1	49	540	25	0.007 (0.002)
GLYDH	49	617	55	0.011 (0.002)
MIP	49	564	84	0.024 (0.003)
CDHH[a]	30	172	19	0.021 (0.006)
CDRF[b]	49	673	121	0.027 (0.003)
YPL109	49	611	18	0.004 (0.001)
RPB2	49	611	12	0.002 (0.001)
CDSTE3.1	9	572	23	0.010 (0.002)
CDSTE3.2	11	475	112	0.085 (0.008)
CDSTE3.3	14	569	13	0.005 (0.001)
CDPHB1	9	201	6	0.011 (0.004)

[a] Noncoding region in between the *Aα* and *Aβ* subloci.
[b] Noncoding region flanking the *Aβ* sublocus, see Fig. 4.

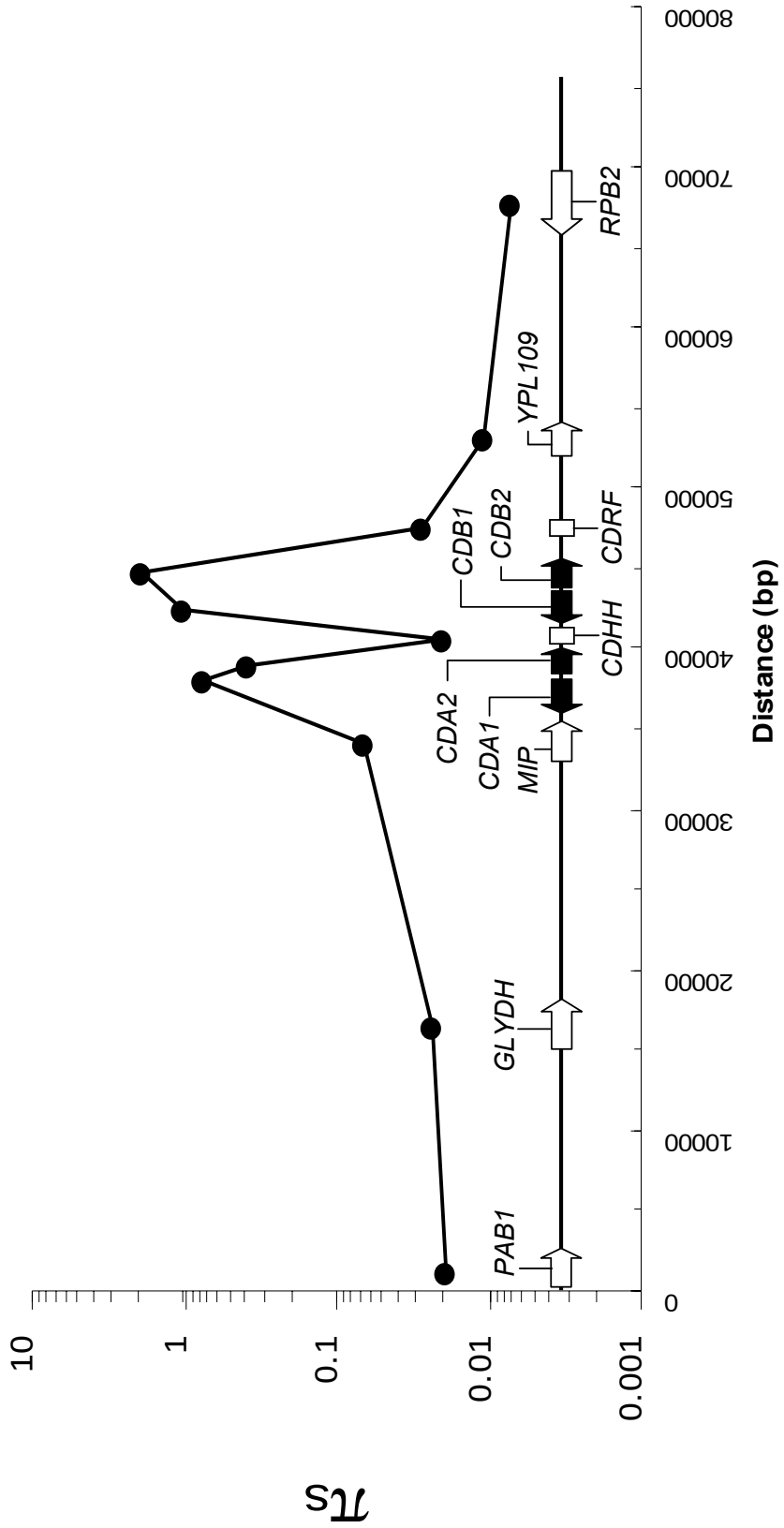


Figure 4. Plot of silent DNA diversity (s) along the *A* mating-type chromosomal region of *C. disseminatus*. The gene map is superimposed below the plot and indicates the position and direction of transcription of the genes in the region. Noncoding loci are shown as white boxes. Data are from the 49 homokaryotic ingroup isolates. The values for the non mating-type genes include intron sequences.

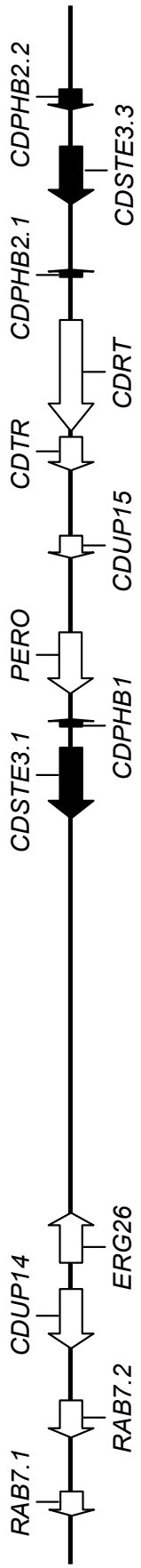


Figure 5. Gene map of the *CDSTE3.1* and *CDSTE3.3* chromosomal region. Arrows indicate the direction of transcription. Genes were identified using BLASTX searches with a cutoff P-value of 10^{-4} (Supplementary Table 2). Genes homologous to other mating-type genes from homobasidiomycetes are shown as black arrows. Two putative transmembrane pheromone receptors (*CDSTE3.1* and *CDSTE3.3*) and three small peptide pheromones (*CDPHB1*, *CDPHB2.1*, and *CDPHB2.2*) were found. *CDUP14* and *CDUP15* are predicted genes with no clear function.

7.4.6. Evolution of the *STE3*-like pheromone receptors

Our analyses of the segregation of the *STE3*-like pheromone receptors demonstrate that these genes are not part of the mating-type locus. A phylogenetic analysis of the basidiomycete pheromone receptor homologues was used to determine if the *STE3*-like genes from *C. disseminatus* actually derive from *B* mating-type receptors of tetrapolar mushrooms. The amino acid sequences of the pheromone receptors f homobasidiomycetes, heterobasidiomycetes, and two Ascomycete outgroups were aligned together with the four *C. disseminatus* putative receptors (see legend of Fig. 6 for GenBank numbers). Only the STE3 domain (pfam02076) sequence region, containing the seven transmembrane helices, was alignable without ambiguity. The maximum likelihood phylogeny estimated using PROTML is shown in Fig. 6. The homobasidiomycete receptors comprised two clades "groups 1 and 2". CDSTE3.1 groups very closely with the two group 1 receptors *S. commun*e BBR2 and *C. cinerea* RCB2.6 and RCB1.3. CDSTE3.2 and CDSTE3.3 are also part of group 1 in a clade with *C. cinerea* RCB3.6 and RCB3.42. Lastly, CDSTE3.4 is nested within the receptors of the group 2 clade. These results demonstrate that the pheromone receptors from *C. disseminatus* have specifically diverged from within the family of mating-type specific pheromone receptors found in other homobasidiomycetes.

The *B* mating-type pheromone receptors of other mushroom species show tremendous DNA and amino acid sequence divergence between alleles (Halsall et al. 2000), presumably due to balancing selection on alleles (May et al. 1999). Thus, if the pheromone receptors of *C. disseminatus* do not encode for proteins involved in the mating incompatibility response, then the genes should not be very polymorphic in natural populations because they are no longer under strong balancing selection. In order to test this we obtained partial sequence data for these genes (*CDSTE3.1*-3) and one putative pheromone gene (*CDPHB1*) from a sample of 9-14 homokaryotic isolates. The results of these analyses are included in Table 5.

The pheromone receptor genes *CDSTE3.1* and *CDSTE3.3* are very tightly linked in the genome (Fig. 5). The observed DNA diversity ( ) at these two loci and the putative pheromone gene *CDPHB1* was similar in comparison with the genes near, but not part of, the *A* mating-type locus (Table 5). *CDSTE3.2* was identified as a unique receptor-like gene because it lacks three of the four introns observed in all other homobasidiomycete receptors. Variation at *CDSTE3.2* suggests it may be a pseudogene because three of eleven sequences contained transcripts predicted to be interrupted by stop codons. Furthermore, the amount of DNA diversity ( ) at *CDSTE3.2* was higher than average (0.086) for non-mating-type gene regions (Table 5). For two homokaryotic strains, PCR amplification using specific primers for *CDSTE3.2* produced two distinct copies. Separation of the two copies using subcloning into a plasmid produced two different but closely

related sequences. Taken together, the *CDSTE3.2* locus appears to be comprised of one or two fast evolving pheromone receptor genes or pseudogenes.

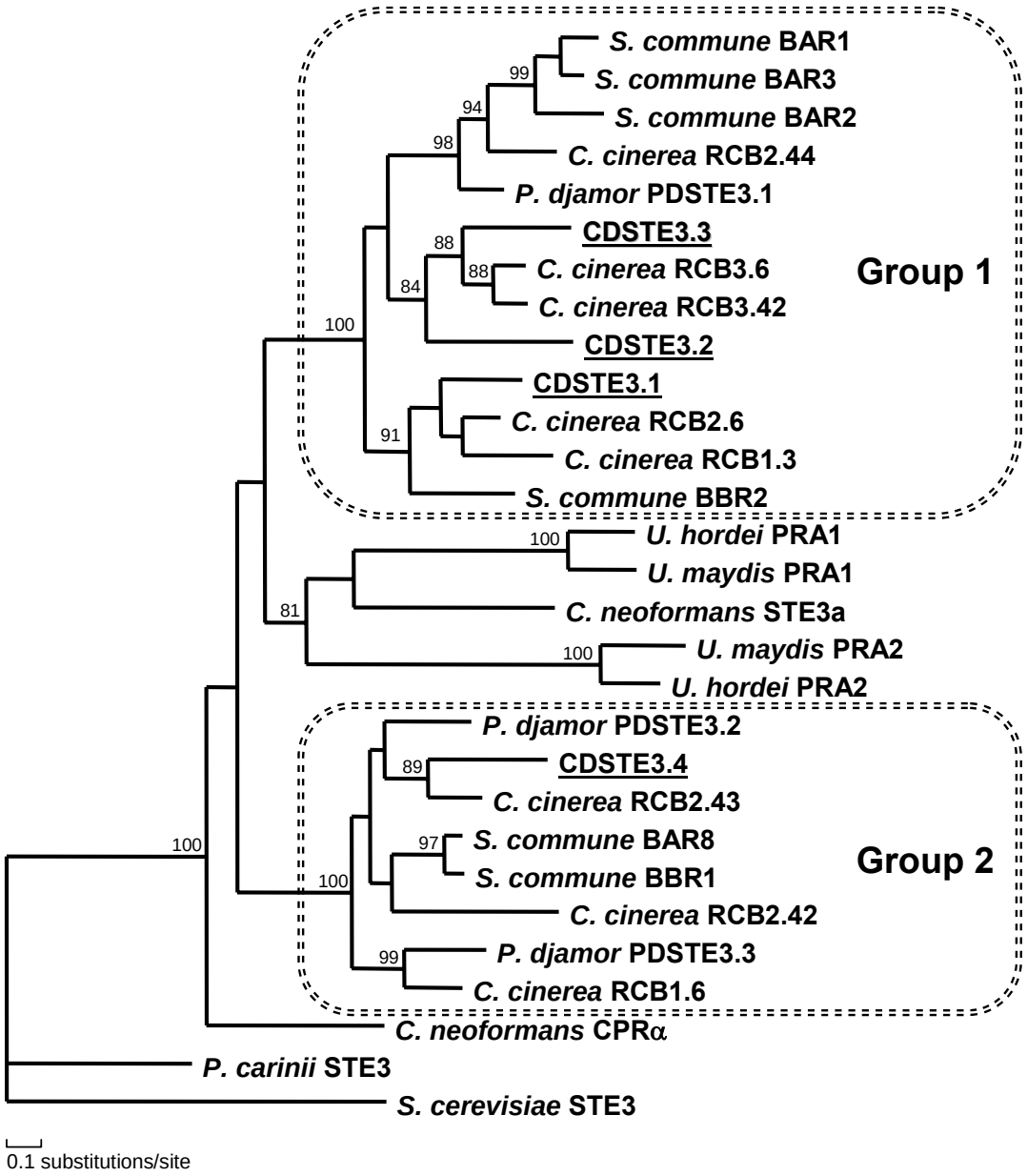


Figure 6. Phylogeny of STE3-like pheromone receptors from basidiomycete fungi. Tree is the maximum likelihood phylogeny estimated using the program PROTML of the MOLPHY v2.3 software package (Adachi and Hasegawa 1996). Numbers above nodes indicate bootstrap probabilities estimated using the RELL method (only shown for nodes above 80%). Two clades of homobasidiomycete pheromone receptors are observed (groups 1 and 2). The four receptor sequences from *C. disseminatus* are underlined. Other sequences are (with GenBank accession numbers in parentheses): *C. cinerea* RCB1.3 (AAO17255), RCB1.6 (CAA71964), RCB2.6 (CAA71963), RCB2.42 (AAF01419), RCB2.43 (AAQ96345), RCB2.44 (AAQ96344), RCB3.6 (CAA71962), RCB3.42 (AAF01420); *S. commune* BAR1 (Q92275), BAR2 (CAA62595), BAR3 (P56502), BAR8 (AAR99618), BBR1 (P78741), BBR2 (AAD35087); *Pleurotus djamor* PDSTE3.1 (AAP57502), PDSTE3.2 (AAP57506), PDSTE3.3 (AAS46748); *Cryptococcus neoformans* CPR (AAF71292), STE3a (AAN75156); *Ustilago maydis* PRA1 (P31302), PRA2 (P31303); *U. hordei* PRA1 (Q99063), PRA2 (AAD56044), *Pneumocystis carinii* (AAG38536); *Saccharomyces cerevisiae* (P06783).

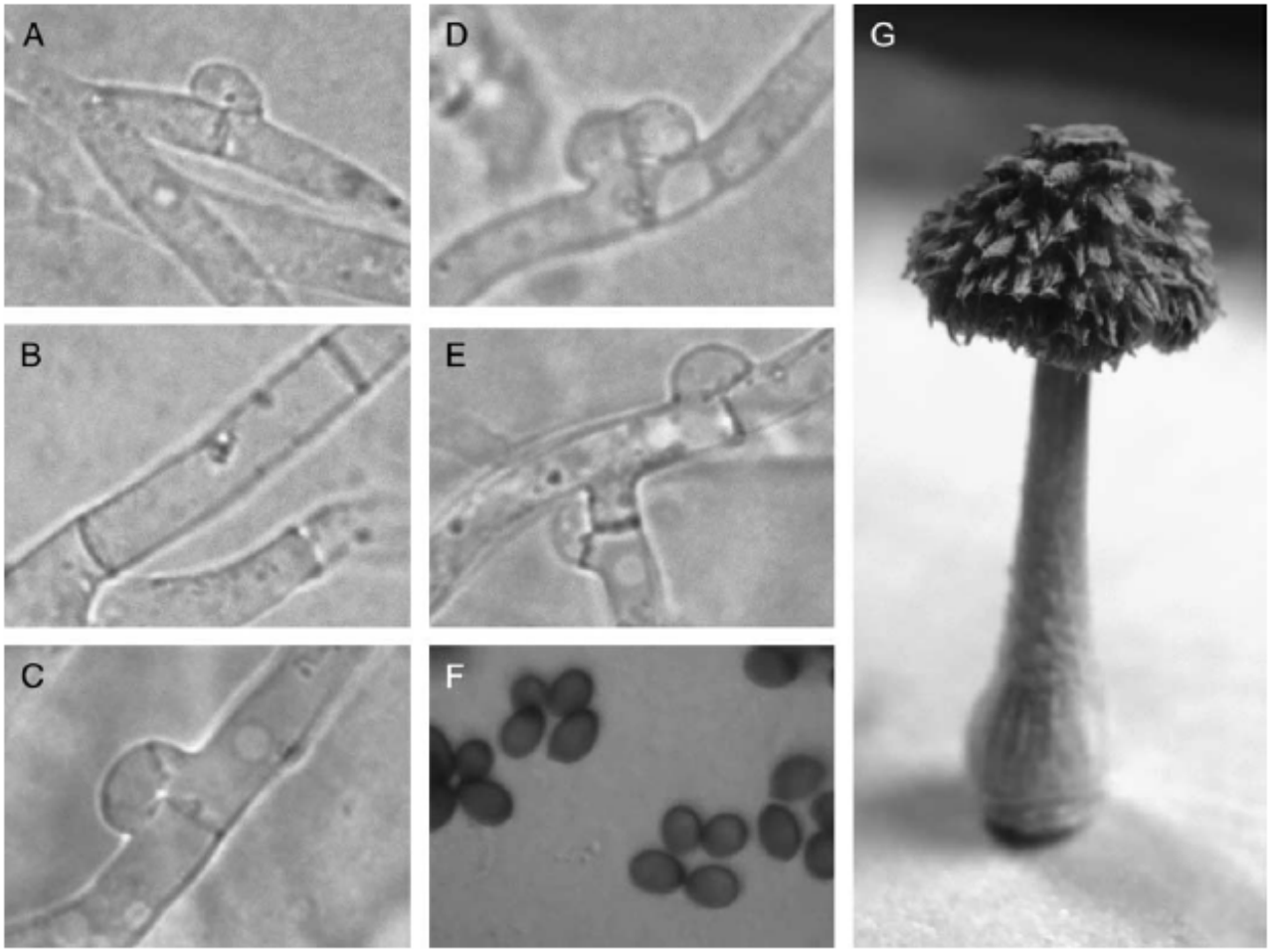


Figure 7. Transformants of *Coprinopsis cinerea A3*, *B1* monokaryon 218. **A.** A transformant of *Coprinellus disseminatus* construct 99.1A with *A* mating type genes has unfused clamps at hyphal septa. **B.** A transformant of C25_e1.10 with *B* homologues from *C. disseminatus* has normal septa. **C.** and **D.** Fused clamp cells and apical clamp cells with enlarged subapical pegs have been observed in the mycelium of transformants of construct 99.1A (with *A* mating type genes) and C25_e1.10 (with *B* mating type genes). Note that all hyphae shown grow from left to right. **E.** Upon mating of the clone shown in **B.** to *A42*, *B1* monokaryon PS004-2, a dikaryon is formed with many fused and unfused clamp cells at hyphal septa. **F.** Under fruiting conditions, the dikaryon produces mature basidiospores on the gills of well developed fruiting bodies **G**.

7.4.7. Function of mating-type gene homologues in *C. cinerea*

When transformed with *A* mating-type genes from its native species, *C. cinerea* monokaryon 218 produces a fluffy mycelium with unfused clamp cells at the hyphal septa. Transformants receiving *B* mating type genes show retarded colony growth and reduced production of aerial mycelium on complete medium. Transformants receiving both *A* and *B* mating type genes grow fluffy with some septa having fused clamp connections. At other septa the apical cell displays an unfused clamp while the subapical side produces an enlarged peg (Kües et al. 1998, 2002; Badalyan et al. 2004). Phenotypes of strain 218 transformants receiving *C. disseminatus* constructs 99.1A ,

16.3A , 99.1A or 98.2A were indistinguishable from phenotypes of transformants with native *A* mating-type genes (Fig. 7a). Functional expression of plasmids carrying *C. disseminatus* homeodomain transcription factor genes in host monokaryon 218 was efficient, with transformation rates between 40 to 51%, irrespective of whether coming from the *Aα* or the *Aβ* sublocus. Transformants of 218 receiving DNA from clones C25 B2.5 and C25_e.10 containing *C. disseminatus* pheromone and pheromone receptor genes had normal septa (Fig. 7b) and no special growth phenotype on YMG/T complete medium. When *C. cinerea* monokaryon 218 was co-transformed with the *C. disseminatus Aα* and *B* constructs, between 28 to 42% of transformants had fused clamp cells at some septa and apical unfused clamps and subapical pegs at other hyphal septa (Fig. 7c-d).

Transformants of strain 218 containing *C. disseminatus* pheromone receptor genes were crossed with the *C. cinereus B1* mating-type tester strain PS004-2. Nuclear migration was observed in both directions, from and/or into 218 transformants, in 45% and 43% of co-transformants using C25 B2.5 and C25_e.10 DNA, respectively. In most cases, clamp cell production in the formed dikaryons was sparse with the majority of clamps unfused to the subapical cell (Fig. 7e). Native *B* genes have been shown in *C. cinerea* to be active in initiation of fruiting when the *A* mating type pathway is activated and to be active in fruiting body maturation at the stage of karyogamy (Kües et al. 2002). Dikaryons formed between PS004-2 and 218 transformants with *C. disseminatus B* homologues (two from C25 B2.5 transformants and five from a C25_e.10 transformant) with many clamp cells (fused and unfused) initiated fruiting up to completion of fruiting body development and basidiospore maturation (Fig. 7f-g).

7.5. Discussion

We have investigated the genetics behind the bipolar mating system of the common mushroom *Coprinellus disseminatus*. Using mating tests, the segregation of molecular markers, and DNA sequencing of large genomic regions, our data suggest that the mating-type locus is comprised of two pairs of homeodomain transcription factors (Fig. 1). Thus, of the two traditional mating-type loci of homobasidiomycetes, the *A* mating-type (homeodomain transcription factor genes) and the *B* mating-type (pheromones / pheromone receptors), only one of these (*A*) actually functions in determining mating-type. Nonetheless, we have discovered at least four pheromone receptor genes homologous to the *B* mating-type genes of other mushrooms using PCR with degenerate primers (Fig. 6). None of the receptors are mating-type specific and none of them show the population genetic signature of balancing selection (i.e., elevated nucleotide polymorphism, Table 4). Furthermore, the heterologous expression of at least one pheromone receptor/pheromone complex in *C. cinerea* suggests that the B mating-type homologues of *C. disseminatus* do still function in a

manner similar to those of tetrapolar homobasidiomycetes, i.e., they are likely to be involved in controlling clamp cell fusion, subapical peg formation, and nuclear migration.

7.5.1. The switch to a bipolar mating system

Of the three hypotheses put forward by Raper (1966) for the origin of the bipolar mating system in homobasidiomycetes from the tetrapolar system, the hypothesis concerning a chromosomal translocation placing the *A* and *B* mating-type genes in close physical association can be ruled out for *C. disseminatus*. This evidence comes from our analyses of the homeodomain transcription factor genes that show cosegregation with mating-type and a pattern of DNA polymorphism characteristic of other mushroom mating-type genes. In contrast, among the pheromone receptor genes found in the *C. disseminatus* genome no such patterns were observed. These results contrast with the findings in the heterobasidiomycete yeast *Ustilago hordei* (Bakkeren and Kronstad 1994) in which the single mating-type locus is composed of a pheromone gene, a pheromone receptor gene, and a pair of homeodomain genes embedded into a nonrecombining chromosomal segment. Thus, a translocation of the *A* mating-type locus into the *B* mating-type region (or vice versa) was suggested by these data. A similar event must have occurred in the ancestor of the heterobasidiomycete yeast *Cryptococcus neoformans* in which the mating-type locus is a non-recombining gene dense region containing many genes important in mating and pathogenesis, including a few pheromone genes, a pheromone receptor gene, and a homeodomain transcription factor gene (Lengeler et al. 2002; Hull et al. 2002; Hull et al. 2005).

Another hypothesis put forward by Raper (1966) for the origin of bipolar mating systems was that one of the two mating-type loci of a tetrapolar ancestor could mutate to become self-compatible, thus rendering its allelic state meaningless in crosses. The data observed for *C. disseminatus* are generally consistent with this hypothesis. We found the *C. disseminatus* homologues of the *B* mating-type genes of *C. cinerea* through degenerate PCR and cosmid sequencing. Although the identified pheromone receptors appear to be fully functional based on *in silico* predictions, they do not show the characteristic hyper-polymorphism associated with mushroom mating-type genes that are under very strong balancing selection (Table 5).

Raper's final hypothesis that the function of one of the mating-types could be gradually assumed by the other mating-type is not consistent with our data. Using heterologous expression in *C. cinerea*, we were able to demonstrate that the *A* and *B* homologues of *C. disseminatus* have very similar cellular phenotypes when transformed into *C. cinerea* as do the respective native *C. cinerea* genes (Fig. 7), suggesting genetic control of the *A* and *B* pathways have been maintained separately in *C. disseminatus*. At least one of the pheromone receptors must be involved in the same G-protein-coupled *B* locus pathway as in other tetrapolar homobasidiomycetes because they are able

to drive clamp-cell fusion and even fruit body development and sporulation (Fig. 7). It remains to be tested whether the receptors or the pheromone genes are constitutively activating or self-compatible mutants or whether they can interact with the native *C. cinerea B* locus proteins.

The mating-type loci of *C. disseminatus* and that of the bipolar mushroom *Pholiota nameko* Aimi et al. (2005) are similar in that both species appear to utilize homeodomain proteins rather than pheromone receptors to determine mating-type. The fact that these evolutionary independent lineages may have taken the same course in evolving a bipolar mating system from a tetrapolar one could suggest that loss of *B* mating-type function is easier than loss of *A* mating-type function. Such a process could occur through differences in rates of mutation to self-fertility of homeodomain proteins versus pheromone receptors.

7.5.2. The structure and evolution of the mating-type locus

C. disseminatus is the first bipolar mushroom species reported to have a mating-type locus comprised of more than a single subunit. We have termed the two separate subloci *Aα* and *Aβ* to facilitate comparison with the traditionally defined mating-type subloci of *C. cinerea* which they closely resemble. The *Aα* and *Aβ* subloci both encode a pair of divergently transcribed homeodomain genes (Fig. 1); the combination of alleles at the two subloci presumably determines the mating-type specificity of an individual. The total number of mating-types in the species was estimated to be ~123 using a complete crossing experiment of 49 homokaryotic isolates. This value is rather similar to that observed at the *A* mating-type of *C. cinerea*, where 120-164 mating-types are estimated (Raper 1966; May and Matzke 1995). We have observed 7 *Aα* and 8 *Aβ* alleles in *C. disseminatus* based on RFLP patterns with *Msp*I, in small samples of 13 and 16 isolates, respectively (see Fig. 3 for representative gel). These molecular phenotypes suggest a symmetric allele number between subloci. Transformation data in *C. cinerea* show that genes from both subloci initiate clamp cell formation, consistent with the hypothesis that the subloci are functionally redundant and independently contribute to mating type specificity.

Although we isolated four pheromone receptors from one haploid strain of *C. disseminatus* and have demonstrated that they are not part of the mating-type locus, additional copies of the *STE3*-like pheromone receptors may still exist that were not detected by our PCR-based methods. If such additional receptors exist, then they are not likely to be part of the mating-type locus. Balancing selection can elevate the polymorphism of neighboring genomic regions, but the increase in diversity is a negative function of the recombination distance between the region and the actual target of selection (Tian et al. 2002). [MKU-ADH/Hudson & Kaplan would be the better example] For the genomic region surrounding the *C. disseminatus A* mating-type locus (i.e., *MIP*,*CDHH*, and *CDRF* loci), DNA polymorphism (π) is over 0.02 (Table 4), but at distances greater than >10 kb

from the mating-type locus, it appears that sites experience little, if any, elevated DNA polymorphism by linkage to the mating-type locus (Fig. 4). Such a contrast in nucleotide diversity between the mating-type locus and the regions bordering it likely reflects recombination that separates those sites subject to strong balancing selection from sites which evolve in a more neutral manner.

7.5.3. An indispensable role for pheromone receptors in homobasidiomycetes

Four lines of evidence point to a clear origin of *CDSTE3.1* and *CDSTE3.3* receptors from other homobasidiomycete mating-type specific pheromone receptors. One line of evidence is that the chromosomal region containing *CDSTE3.1* and *CDSTE3.3* displays some conserved gene order when compared to the *B* mating-type locus chromosomal region of *C. cinerea* (Fig. 2). Two, phylogenetic analyses place these proteins amongst other mating-type proteins of the model mushroom species (Fig. 6). Third, the genes exert *B* mating-type-typical function in a heterologous species (Fig. 7). Lastly, the receptors appear to be each in close physical proximity with one or two putative peptide pheromones (Fig. 5).

If the genome of *C. disseminatus* contains both active pheromone and receptor genes that are not polymorphic, what cellular function do they perform? As mentioned previously, it is possible that they encode proteins locked into a self-compatible complex, turning on the *B* specific developmental pathway through a MAPK cascade, much as their ancestors did for millions of years of fungal evolution. However, it is seems quite unnecessary to maintain a functional pheromone/receptor system to turn on a signaling cascade that could be readily turned on by constitutive activation of its downstream partner, e.g., mutations in *GPA1* can constitutively activate the pheromone response system in yeast (Banuett 1998). That *C. disseminatus* has maintained an apparently functional pheromone receptor system suggests that either the role of the *B* mating-type receptors is more complex than activating only the MAPK pathway through G-proteins, that the G-proteins interact with more than the pheromone receptors, or that the receptors of *C. disseminatus* have taken on a new functional role.

The homobasidiomycete fungi are unique in that they are the only fungal clade to have evolved a multiallelic pheromone/receptor mechanism to perform incompatibility discrimination between individuals- all other fungi have only a biallelic system. The homobasidiomycete pheromone/receptor systems are also possibly unique because these proteins have not been detected extracellularly (Brown and Casselton 2001). It has been suggested that the pheromone/receptor system of mushroom fungi functions in the recognition between the two compatible nuclei of the dikaryotic cell (Schuurs et al. 1998; Debuchy 1999). This function may be indispensable and an intact pheromone/receptor system might be required for proper dikaryon maintenance in all species,

bipolar or tetrapolar. Support for this idea comes from dikaryons formed between *C. cinerea* 218 transformants containing *B* homologues of *C. disseminatus* and *C. cinerea* monokaryon PS004-2. Fruiting bodies, regulated in *C. cinerea* by both *A* and *B* mating type genes (Kües et al. 2002), are formed only on dikaryons with greater than average fused to unfused clamp cells.

7.6. Acknowledgments

We thank John Hopple, Ursula Peintner, Austen Ganley, Ben de Bivort, Jean-Marc Moncalvo, and Hack Sung Jung for providing additional collections or strains of *C. disseminatus*. Jim Johnson provided helpful discussion and references on the evolution of mating systems in *Coprinus*. Marcy Uyenoyama provided helpful comments on an earlier draft, and we thank her for special help in calculating the estimate and confidence intervals for number of mating-types. This work was funded by a National Science Foundation Dissertation Improvement Grant (DEB-01-05031) and a Graduate Student Fellowship from the Mycological Society of America to TYJ. PS is supported by a PhD scholarship of the Mahasarakham University, Thailand. The Göttingen lab is funded by the German Governmental Foundation for Environment (Deutsche Bundesstiftung Umwelt, DBU).

7.7. References

Adachi, J., and M. Hasegawa, 1996 MOLPHY version 2.3: programs for molecular phylogenetics based on maximum likelihood. Comput. Sci. Monogr. **28:** 1-150.

Aimi, T., R. Yoshida, M. Ishikawa, D. Bao, and Y. Kitamoto, 2005 Identification and linkage mapping of the genes for the putative homeodomain protein (*hox1*) and the putative pheromone receptor protein homologue (*rcb1*) in a bipolar basidiomycete, *Pholiota nameko*. Curr. Genet. **48:** 184-194.

Altschul, S. F., T. L. Madden, A. A. Shaffer, J. Zhang, Z. Zhang *et al.*, 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:** 3389-3402.

Badalyan, S. M., E. Polak, R. Hermann, M. Aebi, and U. Kües, 2004 Role of peg formation in clamp cell fusion of homobasidiomycete fungi. J. Basic Microbiol. **44:** 167-177.

Badrane, H., and G. May, 1999 The divergence-homogenization duality in the evolution of the *b1* mating type gene of *Coprinus cinereus*. Mol. Biol. Evol. **16:** 975-986.

Bakkeren, G., and J. W. Kronstad, 1994 Linkage of mating-type loci distinguishes bipolar from tetrapolar mating in basidiomycetous smut fungi. Proc. Natl. Acad. Sci. USA **91:** 7085-7089.

Banham, A. H., R. N. Asante-Owusu, B. Göttgens, S. A. J. Thompson, C. S. Kingsnorth *et al.*, 1995 An N-terminal dimerization domain permits homeodomain proteins to choose compatible partners and initiate sexual development in the mushroom *Coprinus cinereus*. Plant Cell **7:** 773-783.

Banuett, F., 1998 Signaling in the yeasts: an informational cascade with links to the filamentous fungi. Microbiol. Mol. Biol. Rev. **62:** 249-274.

Binninger, D. M., C. Skrzynia, P. J. Pukkila, and L. A.Casselton, 1987 DNA-mediated transformation of the basidiomycete *Coprinus cinereus*. EMBO J. **6:** 835-840.

Bottoli, A. P. F., K. Kertesz-Chaloupková, R. P. Boulianne, J. D. Granado, M. Aebi *et al.*, 1999 Rapid isolation of genes from an indexed genomic library of *C. cinereus* in a novel *pab1*$^{+}$ cosmid. J. Microbiol. Meth. **35:** 129-141.

Borodovsky, M., and J. McIninch, 1993 GenMark: parallel gene recognition for both DNA strands. Comput. Chem. **17:** 123-133.

Brown, A. J., and L. A. Casselton, 2001 Mating in mushrooms: increasing the chances but prolonging the affair. Trends Genet. **17:** 393-400.

Buller, A. H. R., 1924 *Researches on Fungi*, Vol. III. Longmans, Green and Co., London.

Burnett, J. H., 1975 *Mycogenetics*. John Wiley & Sons, London.

Butler, G. M., 1981 Effects of growth retarding environmental factors on growth kinetics and clamp connection occurrence in the dikaryon of *Coprinus disseminatus*. Trans. Brit. Mycol. Soc. **77:** 593-603.

Casselton, L. A., and N. S. Olesnicky, 1998 Molecular genetics of mating recognition in basidiomycete fungi. Microb. Mol. Biol. Rev. **62:** 55-70.

Day, P. R., 1960 The structure of the *A* mating-type locus in *Coprinus lagopus*. Genetics **45:** 641-651.

Debuchy, R., 1999 Internuclear recognition: a possible connection between euascomycetes and homobasidiomycetes. Fungal Genet. Biol. **27:** 218-223.

Fowler, T. J., S. M. Desimone, M. F. Mitton, J. Kurjan, and C.A. Raper, 1999 Multiple sex pheromones and recetors of a mushroom-producing fungus elicit mating in yeast. Mol. Biol. Cell **10:** 2559-2572.

Fowler, T. J., M. F. Mitton, L. J. Vaillancourt, and C. A. Raper, 2001 Changes in mate recognition through alterations of pheromones and receptors in the multisexual mushroom fungus *Schizophyllum commune*. Genetics **158:** 1491-1503.

Granado, J. D., K. Kertesz-Chaloupková, M. Aebi, and U. Kües, 1997 Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. Mol. Gen. Genet. **256:** 28-36.

Halsall, J. R., M. J. Milner, and L. A. Casselton, 2000 Three subfamilies of pheromone and receptor genes generate multiple B mating specificities in the mushroom *Coprinus cinereus*. Genetics **154:** 1115-1123.

Hasegawa, M., and H. Kishino, 1994 Accuracies of the simple methods for estimating the bootstrap probability of a maximum likelihood tree. Mol. Biol. Evol. **11:** 142-145.

Henikoff, S., and J. G. Henikoff, 1992 Amino acid substitution matrices from protein blocks. Proc. Natl. Acad. Sci. USA **89:** 10915-10919.

Hibbett, D. S., and M. J. Donoghue, 2001 Analysis of character correlations among wood decay mechanisms, mating systems, and substrate ranges in homobasidiomycetes. Syst. Biol. **50:** 215-242.

Hiscock, S. J., and U. Kües, 1999 Cellular and molecular mechanisms of sexual incompatibility in plants and fungi. Int. Rev. Cytol. **193:** 165-295.

Hull, C. M., R. C. Davidson, and J. Heitman, 2002 Cell identity and sexual development in Cryptococcus neoformans are controlled by the mating-type specific homeodomain protein Sxi1 . Genes Dev. **16:** 3046-3060.

Hull, C. M., M. -J., Boily, and J. Heitman, 2005 Sex-specific homeodomain proteins Sxi1 and Sxi2**a** coordinately regulate sexual development in *Cryptococcus neoformans*. Eukaryot. Cell **4:** 526-535.

Isaya, G., W. R. Sakati, R. A. Rollins, G. P. Shen, L. C. Hanson *et al.*, 1995 Mammalian mitochondrial intermediate peptidase–structure-function analysis of a new homolog from *Schizophyllum commune* and relationship to thimet oligopeptidases. Genomics **28:** 450-461.

James, T. Y., R. P. Boulianne, A. P. F. Bottoli, J. D. Granado, M. Aebi *et al.*, 2002 The *pab1* gene of *Coprinus cinereus* encodes a bifunctional protein for *para*-aminobenzoic acid (PABA) synthesis: implications for the evolution of fused PABA synthases. J. Basic Microbiol. **42:** 91-103.

James, T. Y., U. Kües, S. A. Rehner, and R. Vilgalys, 2004a Evolution of the gene encoding mitochondrial intermediate peptidase and its cosegregation with the *A* mating-type locus of mushroom fungi. Fungal Genet. Biol. **41:** 381-390.

James, T. Y., S. -R. Liou, and R. Vilgalys, 2004b The genetic structure and diversity of the *A* and *B* mating-type genes from the tropical oyster mushroom, *Pleurotus djamor*. Fungal Genet. Biol. **41:** 813-825.

Ko, K. S., Y. W. Lim, Y. H. Kim, and H. S. Jung, 2001 Phylogenetic divergences of nuclear ITS sequences in *Coprinus* species *sensu lato*. Mycol. Res. **105:** 1519-1526.

Kües, U., and L. A. Casselton, 1992 Homeodomains and regulation of sexual development in basidiomycetes. Trends Genet. **8:** 154-155.

Kües, U., W. V. J. Richardson, A. M. Tymon, E. S. Mutasa, B. Göttgens *et al.*, 1992 The combination of dissimilar alleles of the *Aα* and *Aβ* gene complexes, whose proteins contain homeo domain motifs, determines sexual development in the mushroom *Coprinus cinereus*. Genes Dev. **6:** 568-577.

Kües, U., B. Göttgens, R. Stratmann, W. V. J. Richardson, S. F. O'Shea *et al.*, 1994a A chimeric homeodomain protein causes self-compatibility and constitutive sexual development in the mushroom *Coprinus cinereus*. EMBO J. **13:** 4054-4059.

Kües, U., A. M. Tymon, W. V. J. Richardson, G. May, P. T. Gieser *et al.*, 1994b *A* mating-type factors of *Coprinus cinereus* have variable numbers of specificity genes encoding two classes of homeodomain proteins. Mol. Gen. Genet. **245:** 45-52.

Kües, U., R. N. Asante-Owusu, E. S. Mutasa, A. M. Tymon, E. H. Pardo *et al.*, 1994c Two classes of homeodomain proteins specify the multiple *A* mating types of the mushroom *Coprinus cinereus*. Plant Cell **6:** 1467-1475.

Kües, U., T. Y. James, R. Vilgalys, and M. Challen, 2001 The chromosomal region containing *pab-1*, *mip*, and the *A* mating type locus of the secondarily homothallic basidiomycete *Coprinus bilanatus*. Curr. Genet. **39:** 16-24.

Kües, U., P. J. Walser, M. J. Klaus, and M. Aebi, 2002 Influence of activated *A* and *B* mating-type pathways on developmental processes in the basidiomycete *Coprinus cinereus*. Mol. Genet. Genomics **268:** 262-271.

Kumar, S., K. Tamura, and M. Nei, 2004 MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics **5:** 150-163.

Lange, M., 1952 Species concept in the genus *Coprinus*. Dansk Bot. Ark. **14:** 1-164.

Lengeler, K. B., D. S. Fox, J. A. Fraser, A. Allen, K. Forrester *et al.*, 2002 The mating type locus of *Cryptococcus neoformans*: a step in the evolution of sex chromosomes. Eukaryot. Cell **1:** 704-718.

Marchler-Bauer, A., and S. H. Bryant, 2004 CD-Search: protein domain annotations on the fly. Nucleic Acids Res. **32:** W327-331.

May, G., and E. Matzke, 1995 Recombination and variation at the *A* mating-type of *Coprinus cinereus*. Mol. Biol. Evol. **12:** 794-802.

May, G., F. Shaw, H. Badrane, and X. Vekemans, 1999 The signature of balancing selection: fungal mating compatibility gene evolution. Proc. Natl. Acad. Sci. USA **96:** 9172-9177.

Murphy, J. F., and O. K. Miller, Jr., 1997 Diversity and local distribution of mating alleles in *Marasmiellus praeacutus* and *Collybia subnuda* (Basidiomycetes, Agaricales). Can. J. Bot. **75:** 8-17.

Nicholas, K. B., and H. B. Nicholas, Jr., 1997 *GeneDoc: Analysis and Visualization of Genetic Variation* (http://www.cris.com/~Ketchup/genedoc.shtml).

O'Donnell, S., and M. J. Lawrence, 1984 The population genetics of the self-incompatibility polymorphism in *Papaver rhoeas*. IV. The estimation of the number of alleles in a population. Heredity **53:** 495-507.

Olesnicky, N. S., A. J. Brown, S. J. Dowell, and L. A. Casselton, 1999 A constitutively active G-protein-coupled receptor causes mating self-compatibility in the mushroom *Coprinus.* EMBO J. **18:** 2756-2763.

Olesnicky, N. S., A. J. Brown, Y. Honda, S. L. Dyos, S. J. Dowell *et al.*, 2000 Self-compatible *B* mutants in *Coprinus* with altered pheromone-receptor specificities. Genetics **156:** 1025-1033.

O'Shea, S. F., P. T. Chaure, J. R. Halsall, N. S. Olesnicky, A. Leibbrandt *et al.*, 1998 A large pheromone and receptor gene complex determines multiple *B* mating type specificities in *Coprinus cinereus*. Genetics **148:** 1081-1090.

Pardo, E. H., S. F. O'Shea, and L. A. Casselton, 1996 Multiple versions of the *A* mating type locus of *Coprinus cinereus* are generated by three paralogous pairs of multiallelic homeobox genes. Genetics **144:** 87-94.

Raper, C. A., 1978 Control of development by the incompatibility system in Basidiomycetes, pp. 3-30 in *Genetics and Morphogenesis in the Basidiomycetes*, edited by M. N. Schwalb and P. G. Miles. Academic Press, New York.

Raper, J. R., 1966 *Genetics of Sexuality in Higher Fungi*. Ronald Press, New York.

Raper, J. R., M. G. Baxter, and A. H. Ellingboe, 1960 The genetic structure of the incompatibility factors of *Schizophyllum commune*: the *A* factor. Proc. Natl. Acad. Sci. USA **46:** 833-842.

Raper, J. R., and A. S. Flexer, 1971 Mating systems and evolution of the Basidiomycetes, pp. 149-167 in *Evolution in the Higher Basidiomycetes*, edited by R. H. Petersen. University of Tennessee Press, Knoxville.

Riquelme, M., M. P. Challen, L. A. Casselton, and A. J. Brown, 2005 The origin of multiple *B* mating specificities in *Coprinus cinereus*. Genetics **170:** 1105-1119.

Rozas, J., and R. Rozas, 1999 DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. Bioinformatics **15:** 174-175.

Sambrook, J., E. F. Fritsch, and T. Maniatis, 1989 *Molecular cloning: A laboratory manual*, Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Schuurs, T. A., H. J. P. Dalstra, J. M. J. Scheer, and J. G. H. Wessels, 1998 Positioning of nuclei in the secondary mycelium of *Schizophyllum commune* in relation to differential gene expression. Fungal Genet. Biol. **23:** 151-161.

Stamberg, J., and Y. Koltin, 1973 The organisation of the incompatibility factors in higher fungi: the effect of structure and symmetry on breeding. Heredity **30:** 15-26.

Stevens, R. B., 1974 *Mycology Guidebook*. University of Washington Press, Seattle.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins, 1997 The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:** 4876-4882.

Tian, D., H. Araki, E. Stahl, J. Bergelson, and M. Kreitman, 2002 Signature of balancing selection in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **99:** 11525-11530.

Tusnády, G. E., and I. Simon, 2001 The HMMTOP transmembrane topology prediction server. Bioinformatics **17:** 849-850.

Whitehouse, H. L. K., 1949 Multiple allelomorph heterothallism in the fungi. New Phytol. **48:** 212-244.

Wu, J., R. C. Ullrich, and C. P. Novotny, 1996 Regions in the *Z5* mating gene of *Schizophyllum commune* involved in Y-Z binding and recognition. Mol. Gen. Genet. **252:** 739-745.

Yang, Z., R. Nielsen, N. Goldman, and A.-M. Krabbe Pederson, 2000 Codon-substitution models for heterogeneous selection pressure at amino acid sites. Genetics **155:** 431-449.

Yee, A. R., and J. W. Kronstad, 1993 Construction of chimeric alleles with altered specificity at the *b* incompatibility locus of *Ustilago maydis*. Proc. Natl. Acad. Sci. USA **90:** 664-668.

Yue, C., M. Osier, C. P. Novotny, and R. C. Ullrich, 1997 The specificity determinant of the Y mating-type proteins of *Schizophyllum commune* is also essential for Y-Z protein binding. Genetics **145:** 253-260.

Zhou, B., N. Chen, and Q. Li, 1988 Application of partial restriction procedure in both shotgun and non-random strategies for nucleotide sequencing. Gene **70:** 405-409.

Zolan, M. E., and P. J. Pukkila, 1986 Inheritance of DNA methylation in *Coprinus cinereus*. Mol. Cell Biol. **6:** 195-200.

CHAPTER 8

A constitutively activated Ras-GTPase alters mycelial growth in *Coprinopsis cinerea* and affects *B* mating type-regulated phenotypes in dikaryons and fruiting body development

8.1. Abstract

Ras belongs to the family of small GTPases and acts as a molecular switch, turning on or off downstream signaling pathways in relation to GTP or GDP binding to the protein. A constitutively activated Ras in the basidiomycete *Coprinopsis cinerea* is caused by a point mutation in the codon 19 for glycine, giving rise to the amino acid valine (Ras^{Val19}). A valine at this position impairs the intrinsic GTPase activity of the Ras protein, maintaining it in the GTP-bound state. The ras^{val19} mutant allele has been transformed into different monokaryons of *C. cinerea*. Aerial mycelium is reduced in these transformants but not production of aerial asexual spores (oidia). Mycelium growth is invasive and hyphal branching disturbed. In matings, we observed transfer of ras^{val19} transformed nuclei into compatible monokaryons as well as acceptance of foreign nuclei by the ras^{Val19} transformants. However, the dikaryons formed had unfused clamp cells that often started to re-grow into curly hyphae. As in monokaryons production of aerial mycelium in the dikaryons was suppressed, the general hyphal growth was invasive and the hyphal branching pattern was altered. Fruiting bodies on dikaryons in sectors of a *ras*-disturbed growth phenotype were of reduced size. The primordia had an altered shape and spore formation was partially hampered.

8.2. Introduction

The homobasidiomycete species *Coprinopsis cinerea* is an excellent model mushroom for studying fruiting body development in the basidiomycetes. *C. cinerea* has a short life cycle i.e., the fungus needs 2 weeks from formation of the dikaryotic mycelium to the mature fruiting body. The fungus is easily to handle, i.e., it grows and fruits well on artificial medium under laboratory conditions (Kües 2000), is accessible to classical genetics and can be transformed by DNA (Walser et al. 2001). More than that, the genome of *C. cinerea* has been released by the Broad Institute (see: http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/). *C. cinerea* has two different kinds of mycelia, i.e., the monokaryon and the dikaryon. Monokaryons produce abundant uninucleate haploid mitotic spores (oidia) on specialized oidiophores within the aerial mycelia. The dikaryon can form oidia upon reception of a light signal. During ageing, chlamydospores and the multicellular resting structures called sclerotia arise from the degenerating submerged mycelium of both, mono- and dikaryons. Such asexual structures developing on the monokaryon will germinate into a monokaryon whilst such structures formed at the dikaryon will germinate either into a monokaryon or a dikaryon. The dikaryon furthermore can form the fruiting bodies in which the sexual spores called basidiospores are germinated (Kües 2000; Kües et al. 2002).

C. cinerea is a tetrapolar species that forms basidiospores of 4 different mating type specificities. A single basidiospore germinates into a new monokaryon. When a monokaryon mates with another compatible monokaryon (i.e., of different mating type), the dikaryon is formed. When the nutrients are exhausted and a light signal is given, the dikaryon starts to form the fruiting bodies, a process which is regulated by the *A* and *B* mating type genes. In *C. cinerea*, the products of the *A* mating type genes (homeodomain transcription factors) regulate the clamp cell formation at the dikaryotic hypae and also synchronized nuclear division and septation. Furthermore on the dikaryon, the *A* mating type genes negatively regulate oidia production and induce chlamydosore production and hyphal knot formation as the first step in fruiting (Kües 2000; Kües et al. 2002; Kertesz-Chaloupková et al. 1998; Polak et al. 1997).

A monokaryon that is transformed by a compatible *A* mating type gene (*A*on transformant) is able to form primordia of the fruiting bodies but development is arrested before the stage of karyogamy showing that induced the *A* mating type genes are controlling fruiting body initiation (Kües et al. 2002).

The *B* mating type genes encode lipopeptide mating pheromones and their G-protein-coupled receptors (O'Shea et al. 1998; Kües 2000). The *B* mating type genes control nuclear migration during mating monokaryons and, after mating, fusion of clamp cells with the peg formed on the sub-apical cells of the hyphae of the dikaryon. *A*on *B*on transformants carrying both foreign

A and foreign *B* mating genes are able to develop mature fruiting bodies, unlike *B*on transformants and unlike *A*on transformants. This shows that the *B* genes are also active at the stage of karyogamy (Kües et al. 2002). The *B* mating type genes alone can not induce fruiting, but when the *A* pathway is activated at the same time as the *B* pathways, fruiting body initiation is much more effective, showing that the *B* mating type genes support the *A* mating type genes in their actions.

In *C. cinerea,* sexual reproduction and fruiting body development are regulated not only by the mating type genes but also by various environmental factors such as temperature, light, nutrition (macro- and micro-nutrients) and humidity. Particularly, carbon and nitrogen sources are implicated in the development of fruiting bodies (Matthews and Niederpruem 1972; Kües 2000). Signaling of the carbon level in the environment is thought to be mediated by cyclic adenosine 3′-5′ monophosphate (cAMP) (Uno and Ishikawa 1973a, 1973b). cAMP is a cyclic nucleotide present in any type of organisms. This purine derivative normally occurs in all of the eukaryotic cells. cAMP is a secondary messenger mediating signals from extracellular to intracellular (Kronstad et al. 1998).

cAMP signaling plays important roles in the sexual reproduction and morphology of the plant and human pathogenic basidiomycetes, such as *Ustilago maydis* and *Cryptococcus neoformans*, respectively (Regenfelder et al. 1997; Alspaugh et al. 2002). cAMP triggers fruiting body development in various mushrooms such as *C. cinerea* and *Schizophyllum commune* (Uno and Ishikawa 1973a; Yamagishi et al. 2004). Uno and co-workers found that each stage of the fruiting development in *C. cinerea* had different cAMP levels. cAMP was found accumulated at the stages of secondary hyphal knot formation and decreased in later stage of fruiting body development (Uno and Ishikawa 1973a).

A monokaryotic mutant which carries a constitutive active *fis*^{c} (Fruiting Inducing Substance) copy, a mutated allele of the *fis* gene, induced fruiting body development in *C. cinerea* without mating. The cAMP level in this mutant was found to be increased (Uno and Ishikawa 1973a). Another mutant of *C. cinerea* (*fis*$^{+}$) forms mushrooms upon addition of cAMP to the medium (Uno and Ishikawa 1971).

In *U. maydis*, cAMP stimulates protein kinase A (PKA) and G protein controlled signalling pathways and it is controlling sexual development (Kronstad et al. 1998; Regenfelder et al. 1997; Dürrenberger et al. 1998). Fungi employ cAMP signalling in different processes including the control of cellular differentiation, sexual development, and virulence in addition to the monitoring of nutritional status and stress. The cAMP pathway influences transcription and cell cycle progression (Kronstad et al. 1998). In the ascomycetes yeast *Saccharomyces cerevisiae,* an increase in the activity of PKA correlates with sensitivity to environmental stress, growth defects on carbon sources other than glucose, transient arrest at G1 in the cell cycle, block in sporulation, and

stimulated pseudohypal growth. Two G-proteins, Ras2p and Gpa2P are influenced by cAMP levels *via* regulation of adenylate cyclase (adenyle cyclase, CA) in *S. cerevisiae* (Kronstad et al. 1998; Kübler et al. 1997; Xue et al. 1998).

Ras proteins are the molecular switches in various cellular signalling pathways, that cycle between an active and inactive form (Fig. 1). Ras is active when a GTP (guanosin-5´-triphosphate) molecule is bound to it, and it is inactive in a GDP (guanosin-5´-diphosphate) bound form. To active Ras, the GDP molecule on the GDP-form is exchanged to GTP by the guanine exchange factor (GEF), whereas the GTP bound from is inactivated by transforming the GTP to GDP by the Ras GTPase–Activating Protein (GAP).

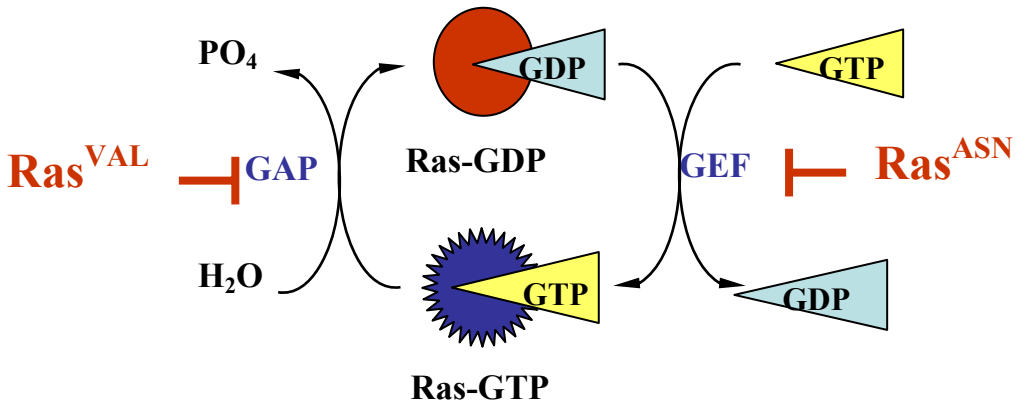


Figure 1. Regulation of the Ras GTPase function (after Bottoli 2001; Boguski and McCormick 1993; Toda et al. 1985; Farnsworth and Feig 1991).

The activated Ras stimulates the adenylate cyclase (AC) to produce cAMP from ATP molecule (Bivona and Philips 2003; Downward 2003). The Ras family of GTPase proteins has been shown to control morphogenesis in many organisms, including several species of pathogenic and basidiomycetes fungi (Fortwendel et al. 2005; Schubert et al. 2006; Waugh et al. 2002). Interestingly, the Ras protein regulates the multiple cellular signaling pathways, which control growth, cellular proliferation and differentiation. In *Aspergillus fumigatus,* for example, deletion of the *rasB* gene caused decreases in spore germination and growth rates and germinated colonies had an irregular hyphal morphology (Fortwendel et al. 2005). In *C. neoformans,* strains which have a deletion of the *ras1* gene were unable to mate under nutrient starvation due to the failure to produce the mating filaments in response to a compatible mating haploid partner. A mutant strains also fail to trigger the mating filament formation in the compatible wild type (Alspaugh et al. 2000; Waugh et al. 2002). In *S. commune,* Yamagishi and his co-workers found that the *ras* gene has a repressing

effect on colony growth in dikaryons and on fruiting body formation. cAMP levels were not significantly altered, suggesting that the *ras* gene may not function through the cAMP signaling pathways (Yamagishi et al. 2004). Schubert and her colleagues found that the *Gap1* gene for the Gap1 protein of Ras regulates hyphal growth orientation, the growth rates of monokaryons and dikaryons and various steps in sexual development (clamp cell fusion, the structure of the fruiting body with altered grills and basidiospore formation) (Schubert et al. 2006). In this study, I use a constitutively activated *ras* allele (*ras* ^{Val19} and a constitutively maintained ras allele (*ras*^{Asn24}) created by Bottoli (2001), to study effects of Ras in developmental processes of *C. cinerea*. A constitutively activated Ras is obtained by a single amino acid replacement at a defined position in the N-terminal GTP/GDP binding domain (Toda et al. 1985). In the *C. cinerea* Ras protein, this is a glysine to valine exchange at amino acid position 19 (Bottoli 2001). Its effect is that GTP is constantly bound to Ras due to a failure of function the GAP protein to transform it into GDP. In the constitutively inactivated form of the Ras protein, an amino acid exchange from Gly to Asn at position 24 (Bottoli 2001) blocks the activation of GEF, resulting in constant binding of GDP to Ras (Farnsworth and Feig 1991).

8. 3. Materials and Methods

8.3.1. Strains, culture condition, transformation and microscopy

For oidia harvesting and in mating reactions, *C. cinerea* monokaryons 218 (*A3 B1 trp1.1,1,6, bad*), FA2222 (*A5 B6 trp1.1,1.6*), PS001-1 (*A42 B42*), PG78 (*A6 B42, pab-1, trp1.1,1,6*) and AT8 (*A43 B43, trp-3, ade-8*) (Kertesz-Chaloupková et al. 1998 and chapter 3 of this thesis) were grown at 37 °C on complete medium YMG/T (Granado et al. 1997). Oidia were used in co-transformation as described by Granado et al. (1997). The *trp1*$^{+}$ plasmid pCc1001 (Binninger et al. 1987) was used for selection of positive transformants. Plasmid pRAS^{wt} contains the wild type *ras* gene of *C. cinerea* homokaryon AmutBmut cloned into pBluescript KS^{-}, plasmid pRAS ^{Val19} the allele for the constitutively activated Ras protein and plasmid pRAS ^{Asn24} the allele for the constitutively inactivated Ras protein (Bottoli 2001).

Monokaryons 218 and FA2222 were used in transformations. Transformants were picked onto minimal medium (MM) (Granado et al. 1997) and grown at 37 °C in the dark. Colony growth was observed on MM by visual inspection and hyphal structures were observed under the microscope. Colony growth on plates was marked every day and increase of growth was measured with a ruler. Similarly, transformed monokaryons were also grown on complete medium for observing growth rates of colonies. Small colonies were photographed using A Color-view MegaPixel-camera (Imaging System program, Münster, Germany) camera assembled on a Stemi

2000-C Zeiss, binocular (Göttingen, Germany), that was linked to a computer equipped with analySIS® Soft ware program (Münster, Germany). Lager colonies were photographed by Color-veiw MegaPixel-camera equipped with the JENOPTIK lens (Jena, Germany).

To further study growing of hyphae, sterile cellophane was laid onto minimal medium before growth at 37 °C. Small pieces of cellophane with the edges of the colonies were cut and laid onto a microscope slide for observation using Zeiss Axiophot photomicroscope equipped with Color view soft image system camera.

Mating between 218 and FA2222 transformants and between a transformant and monokaryons AT8, PG78 and PS001-1 was done as described by Walser et al. (2001). Mating between two strains was scored by outgrowth of dikaryotic hyphae of the outer edges of the two mating partners (reflecting donation of nuclei to and acception of nuclei from the mating partners and by outgrowth of dikaryontic mycelia from in between the colonies (Walser et al. 2001). Dikaryotic growth was confirmed by observation of clamp cells (Kües 2000).

For observation of colony and hyphal growth, dikaryons were grown at 37 °C on YMG/T without and with cellophane, respectively. Colonies on plates were photographed as described above. For fruiting, dikaryons were grown at 37 °C under constant dark on YMG/T agar before transforming the plates into 25°C, 80-90% humidity, 12 h dark/12 h light cycle (Granado et al. 1997).

Fruiting structures were observed and photographed under the binocular before and after cutting in half. Gills were taken from developing primordia, squashed on a glass slide and the nuclei in the basidia were DAPI (4′,6-diamidine-2-phenylindale dihydrochloride; Boehringer Mannheim, Germany) stained following the protocol as described by Polak et al. (1997).

8.3.2. Statistical Analysis

Data are presented as means ± S.E.M. Significant differences between the controls and treatment groups were analyzed by one-way ANOVA (Dunnett's post-test) following with the multiple *t*-test comparison (Prism; GraphPad, San Diego, CA, USA). The significant differences between each control and treatment group under the different experimental factors i.e., growth rates on YMG/T *vs.* MM were analyzed by two-way ANOVA (Bonferroni post-tests) (Prism; GraphPad, San Diego, CA, USA). *P* values < 0.05 were considered significant.

8.4. Results

8.4.1. *Ras* ^{Val19} affects the mycelia of monokaryons

1 µg pRAS^{Wt}, pRAS^{Asn24} and pRAS^{Va119}, respectively, were co-transformed with each 1 µg of pCc1001 into monokaryons 218 and FA2222. In addition, transformations were performed with the two strains with 1 µg of pCc1001 alone. In case of monokaryon 218 and pRAS^{Va119} on regeneration agar, there were two mycelial colony phenotypes, i.e., normal, well growing transformants appearing on day 4 after transformation with a colony diameter of several mm (2-3mm) and transformants growing very condensed to a colony size of only about 1 mm or less (not shown, but see Bottoli 2001 for comparison of alike 218 transformants). The dense clones in this study occurred in a frequency of 11%. No such colonies were observed in the other transformations of monokaryon 218 (table 1). These results confirmed earlier observations by Bottoli (2001).

In case of monokaryon FA2222 on regeneration agar, similarly two types of colonies, one normal growing and one condensed growing, were observed on regeneration agar. The condensed growing colonies occurred at a frequency of 21%. Transformants of both experiments were transferred onto MM (10 colonies per plate) and incubated at 37 °C for 36 h.

Table 1 Colony phenotypes observed upon transformation of monokaryon 218

Plasmid (s)	Colonies		
	Total obtained	**With normal growth**	**With condensed growth**
pCc1001	61 (100%)	61 (100%)	0 (0%)
pCc1001 + pRAS^{Wt}	39 (100%)	39 (100%)	0 (0%)
pCc1001 + pRAS^{Asn}	98 (100%)	98 (100%)	0 (0%)
pCc1001 + pRAS^{Val}	109 (100%)	97 (89%)	12 (11%)

Table 2 Colony phenotypes observed upon transformation of monokaryon FA2222

Plasmid (s)	Colonies		
	Total obtained	**With normal growth**	**With condensed growth**
pCc1001	42 (100%)	42 (100%)	0 (0%)
pCc1001 + pRAS^{Wt}	24 (100%)	24 (100%)	0 (0%)
pCc1001 + pRAS^{Asn}	23 (100%)	23 (100%)	0 (0%)
pCc1001 + pRAS^{Val}	41 (100%)	32 (79%)	9 (21%)

pCc1001 transformants (Fig. 2 A, C), transformants from the *ras*^{Wt} experiments, and transformants of *ras* ^{Asn} experiments that grew normal on regeneration agar gave the normal mycelial phenotype of monokaryon 218 and FA2222, respectively also on MM. However, those of the *ras*^{Val19} transformants that grew densely on regeneration medium had also an altered colony and hyphal growth on minimal medium (Fig. 2 B, D), in contrast to those that had already on regeneration agar a normal phenotype. *ras*^{Val19} transformants grew slower and more densely than control transformants.

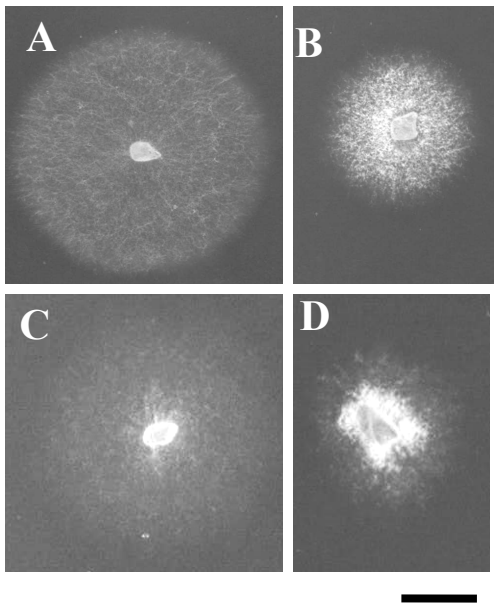


Figure 2. *ras*^{Val19} transformants grown on minimal medium at 37 °C for 36 h **(A).** pCc1001 transformant of monokaryon 218, **(B).** *ras*^{Val19} transformant of monokaryon 218, **(C)**. pCc1001 transformant of monokaryon FA2222 and **(D)**. *ras*^{Val19} transformant of monokaryon FA2222. The size bar corresponds to 1 cm.

A selection of pCc1001 transformants of both transformations (5 clones per transformation), and 218/Ras^{Val19} transformants and FA2222/Ras^{Val19} transformants with a small condensed colony phenotype (7 clones per transformation) were transferred onto new MM plates and also onto YMG/T plates. This time, one inoculum per plate were placed into its middle. Colonies were grown at 37 °C in the dark until plates were fully grown. The growth rates of 218/Ras^{Val19} transformants and FA2222/Ras^{Val19} transformants were reduced on both MM and YMG/T (Fig 3. and 4). It took about 10 to 15 days to fully grow on MM plates, respectively (Fig 3. and 5), and 15 to over 20 days as well as on YMG/T (Fig. 4). For comparison, pCc1001 transformants took 6 to 7 days to grow fully cover a plate with MM and with YMG/T medium (Fig. 3 to 5). The growth rates of a pCc1001 transformant on MM and YMG/T were 0.92 and 0.65 cm/day, respectively. A 218/Ras^{Val19} transformant grew only 0.30±0.11 cm/day and a FA2222/Ras^{Val19} transformant 0.44±0.18 cm/day on MM medium. The 218/Ras^{Val19} transformant grew 0.34±0.14 cm/day and the FA2222/Ras^{Val19} transformants 0.15±0.08 cm/day on YMG/T medium, respectively (Fig 3 and Fig 5).

In comparison, the growth speed of dikaryon made from the some monokaryons was less dramatically affected compared to a dikaryon of pCc1001 transformants. The growth rates of dikaryon 218/ras^{Val19} X monokaryon PS001-1, FA2222/ras^{Val19} X monokaryon PS001-1 and 218/ras^{Val19} X FA2222/ras^{Val19} were 1.01±0.32, 0.72±0.26, 0.66±0.31 cm/day, respectively, whereas the growth rate of the control 218/pCc1001 X FA2222/ pCc1001 was 1.06 ± 0.35 cm/day.

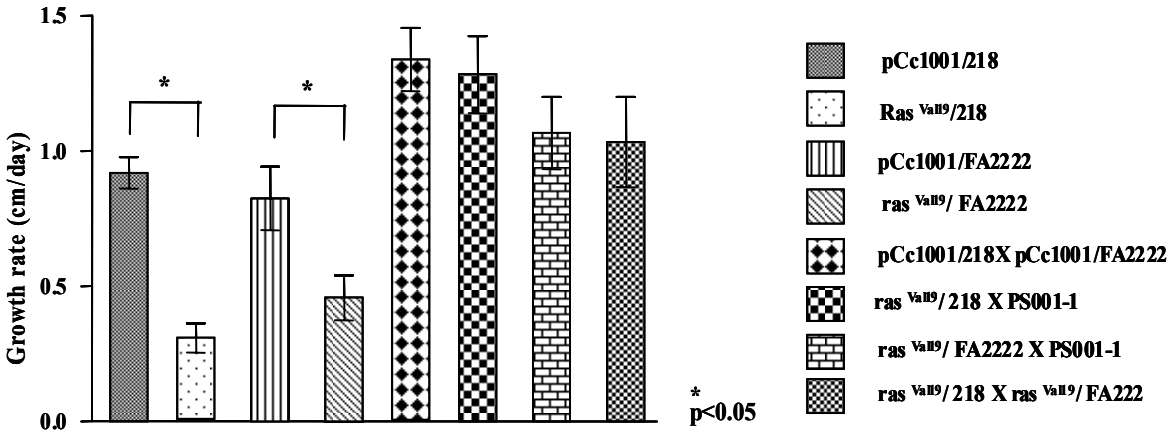


Figure 3. Growth rate (cm/day) of transformed monokaryons and dikaryons on MM. Plates were incubated at 37 °C in a black ventilated box. Each strain was grown and analyzed in duplicate growth differences between two transformants marked by star (*) was considered significant (*P<0.05).

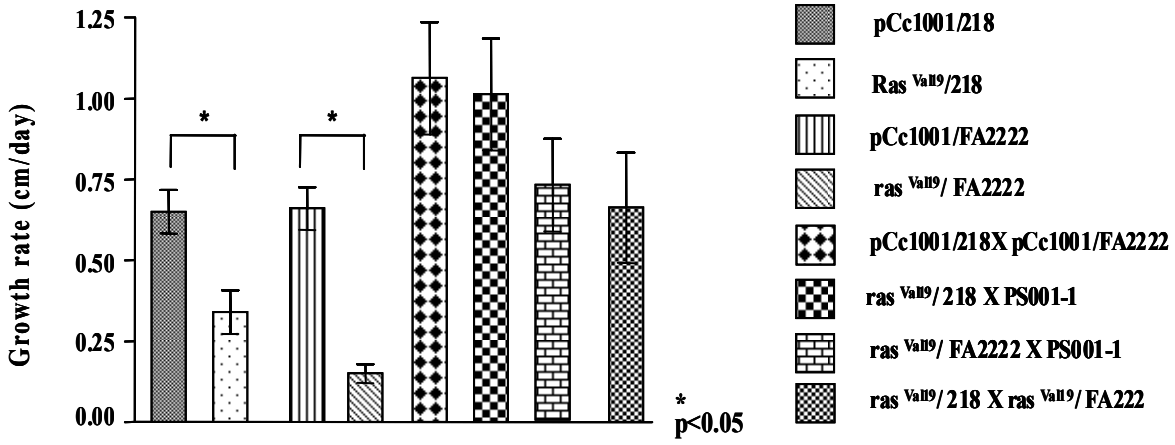


Figure 4. Growth rate (cm/day) of transformed monokaryons and dikaryons on YMG/T. Plates were incubated at 37 °C in a black ventilated box. Each strain was grown and analyzed in duplicate growth differences between two transformants marked by star (*) was considered significant (*P<0.05).

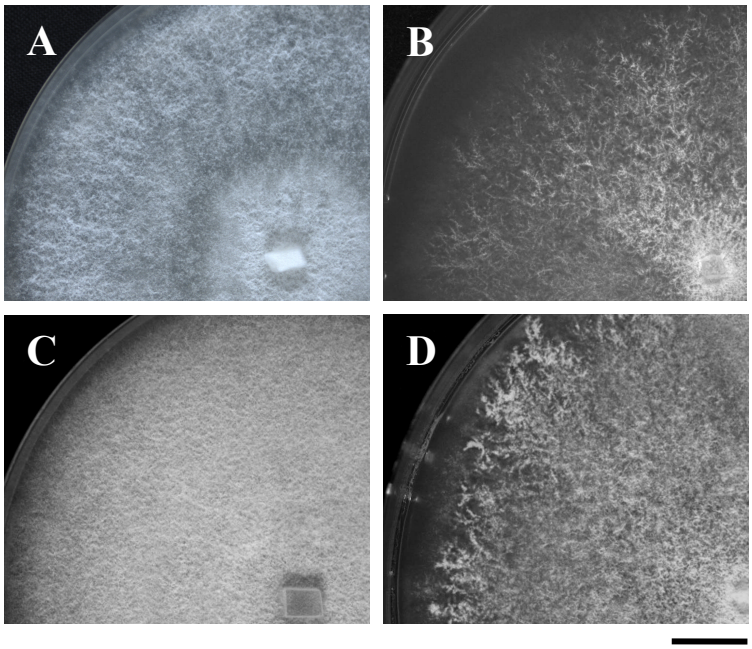


Figure 5. Ras^{Val19} affects the growth of the vegetative mycelia of 218 and FA2222 monokaryons. (**A)** and **(C):** pCc1001 transformants of monokaryon 218 and monokaryon FA2222, respectively, were grown for 7 days at 37 °C on MM in the dark before photographs of the plates were taken. (**B)** and **(D):** *ras* ^{Val19} transformants of monokaryon 218 and monokaryon FA2222, respectively, were growth for 15 days on MM in the dark before photographs of the plates were taken. Note the irregular growth of the colonies of the *ras* ^{Val19} transformants. The size bar corresponds to 1 cm.

Production of aerial mycelium of the 218/r*as*^{Val19} and FA2222/*ras*^{Val19} monokaryons on YMG/T was reduced (not shown). Moreover, hyphal growth was invasive to the agar causing the agar surface to bend. When the 218/r*as*^{Val19} transformants were fully grown on YMG/Tat 37 °C under dark conditions, we observed one or two days later primary hyphal knots [small hyphal aggregates generated by local intense branching Boulianne et al. 2000)] formed in the aerial mycelium (not shown). Within 2 further days they transformed into pigmented sclerotia, multiple cellular resting bodies with an outer melanised rind and an inner brown-melanised tissues known as medulla (Kües et al. 2002). Sclerotia also occurred in pCc1001 controls of monokaryon 218 as described before (Kües et al. 1998). However in the *ras* ^{Val19} transformants, there were also larger mycelial bodies (Fig. 6). These were formed by mycelial growth around liquid drops upon which these became turbid mycelial balls (indicated by the arrow in Fig. 6) and eventually were brown-coloured (Fig 6). Bottoli (2001) also observed these structures in transformants of ras ^{Val19} monokaryon 218 and reported they would be filled by oidia. In transformants of monokaryon FA2222, sclerotia or larger mycelia bodies were not observed, neither in the pCc1001 colony, nor in colonies of pRAS ^{Val}. The results with control transformants confirm earlier observations by Kües et al. (1998).

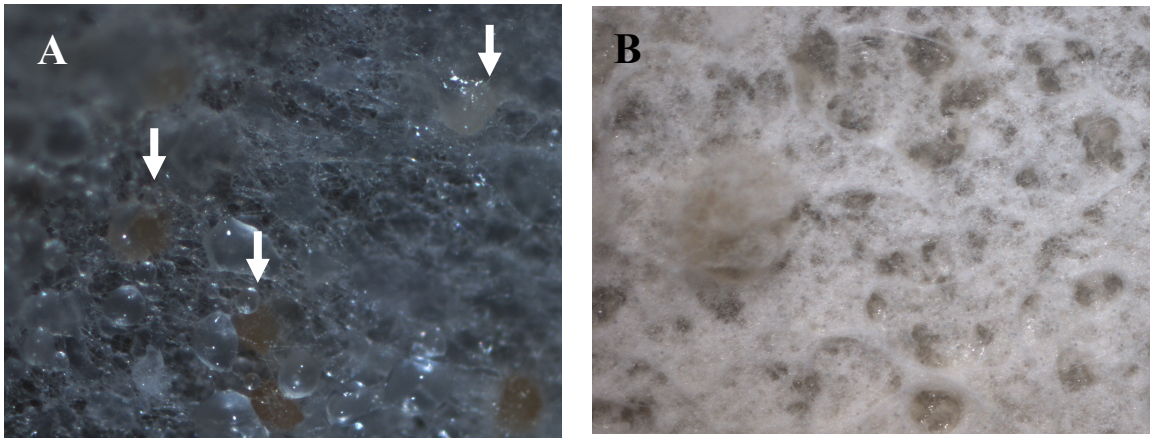


Figure 6. (**A**). Larger mycelial bodies occurred on 218/*ras* ^{Val19} transformants, (**B**) No such larger mycelial bodies were formed on FA2222/*ras* ^{Val19} transformants.

Hyphal growth was observed after growing 218/r*as*^{Val19} and FA2222/*ras*^{Val19} transformants and controls on MM and YMG/T plates with cellophane, at 37 °C. When hyphal growth of *ras*^{Val19} and pCc1001 transformants were compared (Fig. 7 and Fig. 8), we detected that *ras*^{Val19} had an effect on the orientation of hyphal growth. Hyphae of the 218/r*as*^{Val19} and FA2222/*ras*^{Val19} transformants were more thin and showed irregular growth with repeatedly changing growth direction and with intense production of many short hyphal branches. In contrast, the controls had the long straight formed growing, thicker hypae with few hyphal branches.

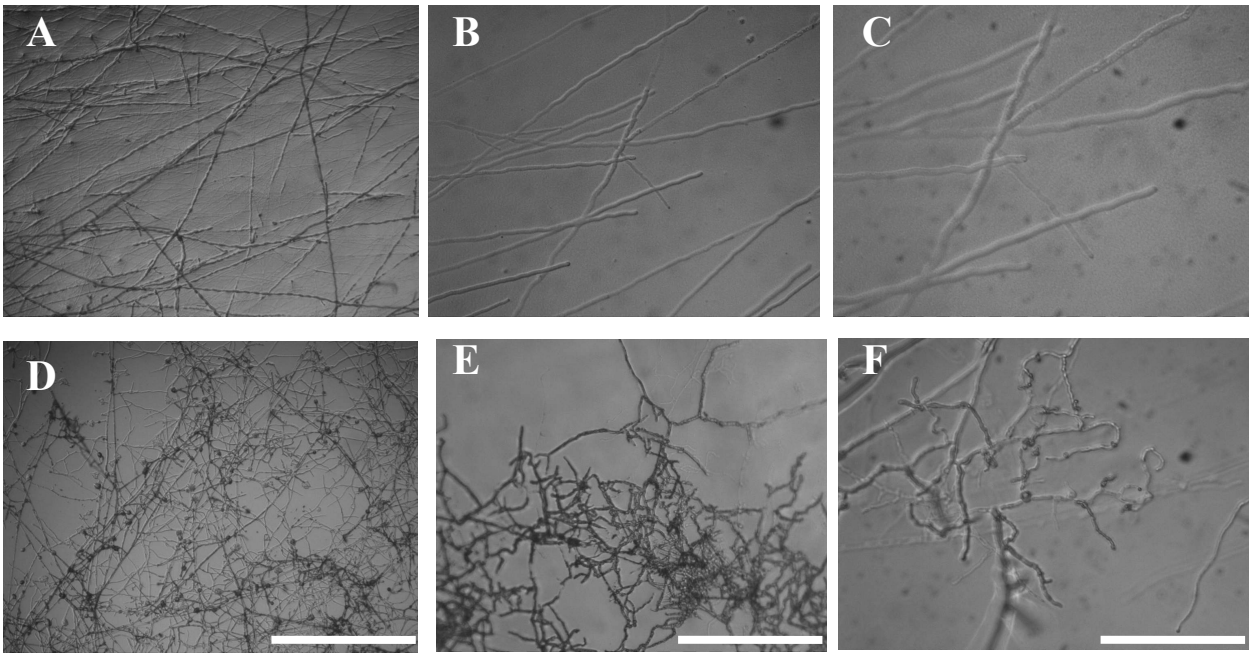


Figure 7. Hyphal branching pattern at the edge of colonies of 218 transformants grown on cellophane MM plates. (**A** to **C**) control (pCc1001), (**D** to **F**): mycelium a *ras*^{Val19} transformant. The pictures were taken in various magnifications (10x, 20x and 40x). Size bars correspond to 200 μm, 100 μm and 50 μm, respectively.

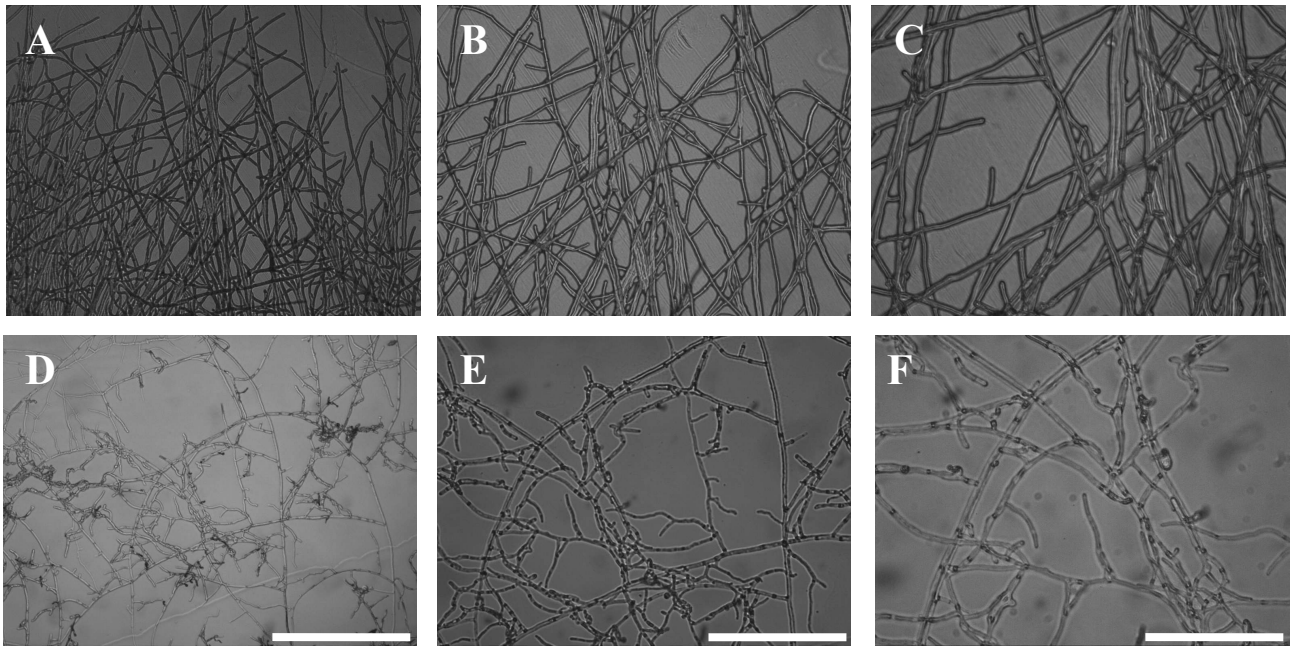


Figure 8. Hyphal branching pattern at the edge of colonies of FA2222 transformants grown on cellophane MM plates. (**A** to **C**) control (pCc1001), (**D** to **F**): mycelium a *ras*^{Val19} transformant. The pictures were taken in various magnifications (10x, 20x and 40x). Size bars correspond to 200 μm, 100 μm and 50 μm, respectively.

8.4.2. Mating of *ras* ^{Val19} transformants

Earlier observation on *ras* ^{Val19} transformants of monokaryon PG78 suggested that *ras* ^{Val19} transformants act well as acceptors of nuclei in mating with other monokaryotic strains (Kües personal communication). Here, we tested in crosses with transformed monokaryons the mating behavior of *ras*^{Val19} transformants, 218/*ras*^{Val19} and FA2222/r*as* ^{Val19} monokaryons were mated at 37 °C with the monokaryotic strains PG78, AT8 and PS00-11. Mating was scored after 5 days for dikaryon formation. 218/*ras*^{Val19} and FA2222/r*as* ^{Val19} transformants behaved as nuclei donors and acceptors with a possible preference for donating nuclei. In addition, there was outgrowth of dikaryotic mycelium from in between mating partners (Table 3, Table 4 and Fig. 9).

Table 3 Behavior of mating of 218/*ras*^{Val19} transformants with *C. cinerea* monokaryons PG78, AT8, and PS001-1

Monokaryons mated with 218/*ras*^{Val19} transformants	**Behavior in mating of 218/*ras*^{Val19} transformants**			
	Total clones mated	**Nuclei donor**	**Nuclei acceptor**	**Outgrowth of dikaryon**
PG 78	7	4	1	2
AT8	1	0	1	0
PS001-1	4	0	1	3

Table 4 Behavior of mating of FA2222/*ras*^{Val19} transformants with *C. cinerea* monokaryons PG78, AT8, and PS001-1

Monokaryons mated with FA2222/*ras*^{Val19} transformants	**Behavior in mating of FA2222/*ras*^{Val19} transformants**			
	Total clones mated	**Nuclei donor**	**Nuclei acceptor**	**Outgrowth of dikaryon**
PG 78	7	4	0	3
AT8	7	2	3	2
PS001-1	5	4	1	4

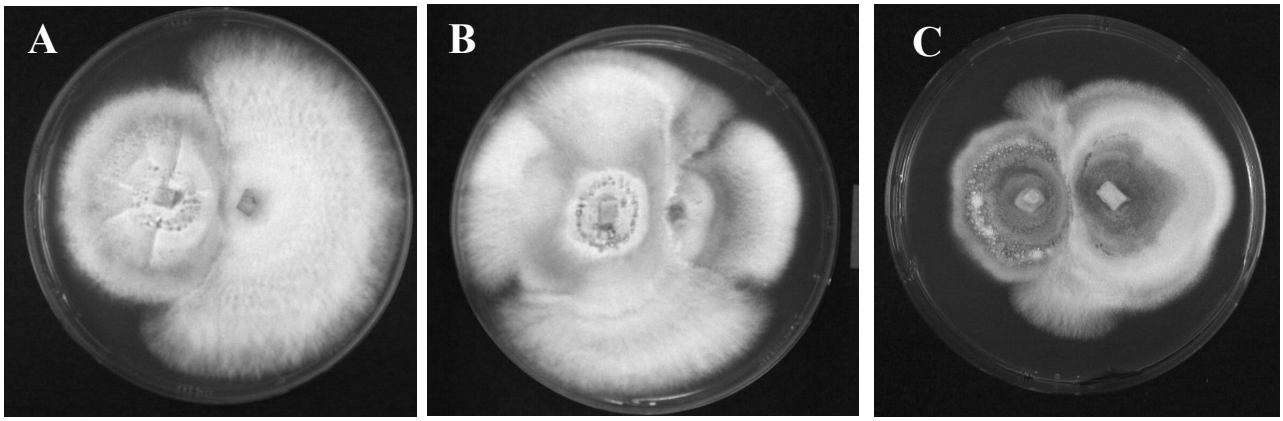


Figure 9. Mating reaction behavior of *ras* ^{Val19} transformants with monokaryons inoculated right, (**A**)**.** In a cross of FA2222/*ras*^{Val19} transformants with monokayon PS001-1, transformant reacted a donor of nuclei as deduced from the out growth of a fluffy mycelium from monokaryon PS001-1 (**B**). With monokaryon PG78, FA2222/*ras*^{Val19} transformant reacted as both nuclei donor and acceptor, note the outgrowth of fluffy dikaryotic mycelium on both colonies inoculated (**C**). In a cross with monokaryon AT8, *ras*^{Val19} transformant of FA2222 neither donated nor accepted nuclei and dikaryon outgrowth occurred in between the two inoculated colonies.

8.4.3. Ras ^{Val19} influences clamp formation

The dikaryons 218/pCc1001 X monokaryon PS001-1, 218/*ras*^{Val19} X monokaryon PS001-1 and FA2222/*ras*^{Val19} X monokaryon PS001-1 were generated by mating between monokaryon PS001-1 either with 218/pCc1001, 218/*ras*^{Val19} or FA2222/*ras*^{Val19}*,* respectively. A single inoculum of each dikaryon 0.5 x 0.5cm in size was placed at the center of YMG/T plate and then incubated at 37 °C for 4-6 days. The dikaryotic mycelia which are carrying the *ras*^{Val19} grew somewhat slower when compared with the controls (Fig. 10). The dikaryons formed an abnormal that growing mycelial phenotype and the normal dikaryotic mycelial area (Fig. 10), like the "*flat*" phenotype in *S. commune.* Also in semi-*ras*^{Val19}/FA2222 dikaryon, hyphal growth was invasive into the agar with the consequence of bending the agar surface (Fig. 9 A). However, upon longer growth, abnormal dikaryotic mycelia reverted to normal dikaryotic mycelial phenotype (see Fig 10) clamp cell formation was observed in both normal and abnormal mycelial areas. However, there were many abnormal unfused clamp cells and some normal fused clamp cells have found in both areas.

One or more clamp cells developed apical and sub-apical to a septum, they growth all possible direction and did of the not stop growth that continued into a hyphae. Normally, there is only one clamp cell formed per septum. Most grow backwards compared to the control (218/pCc1001 X PS001-1) hyphal growth direction (A and D).

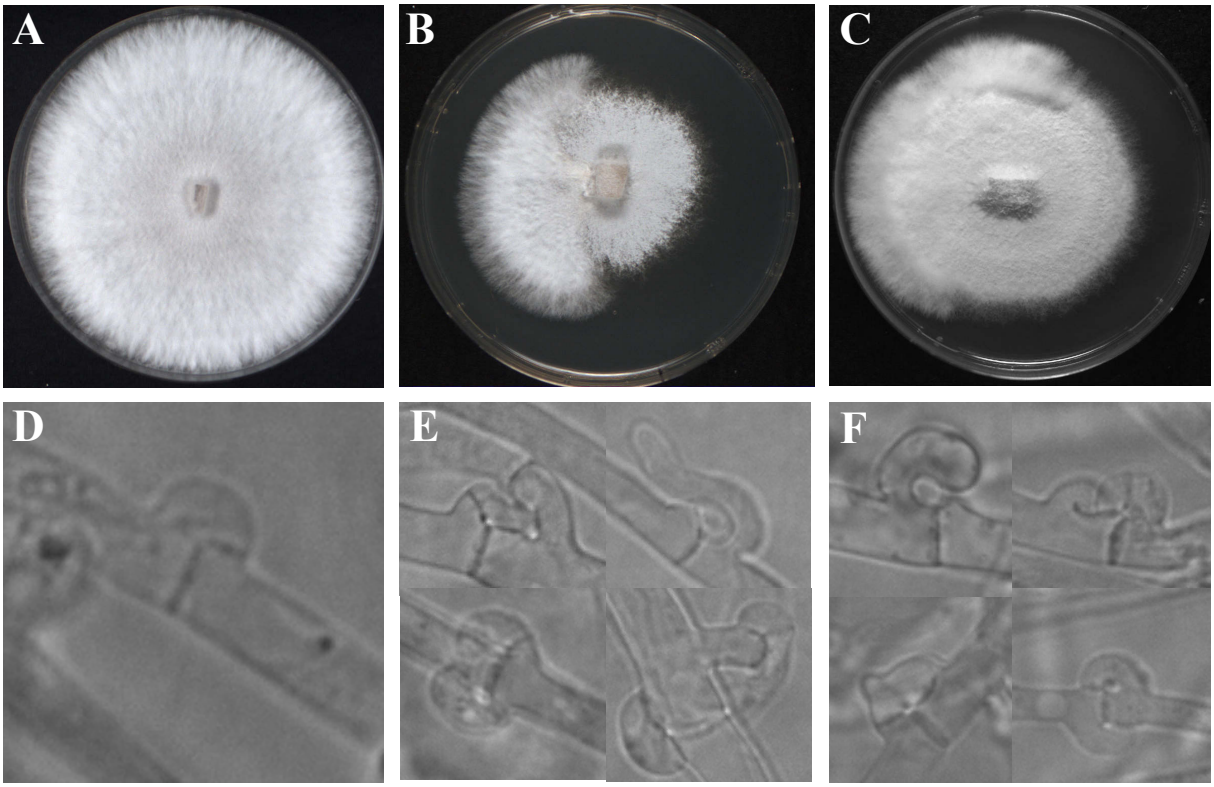


Figure 10. Colony growth and clamp cells formation of dikaryons. (**A** and **D**) control dikaryon 218/pCc1001 X PS001-1. (**B** and **E**) dikaryon 218/*ras*^{Val19} X PS001-1 a "semi-*Ras*^{Val19} dikaryon", and (**C** and **F**) FA2222/*ras*^{Val19} X PS001-1, also "semi-*Ras*^{Val19} dikaryon". Note that colony growth in "semi-*Ras*^{Val19} dikaryon" dikaryon is retarded (compare **B** and **C** with **A**) and there is sector formation with outgrowth of fluffly mycelium. Control dikaryons have fused clamp cells (**D**). Unlike "semi-*Ras*^{Val19} dikaryons" where clamp cell fusion rarely takes places, independently of whether the mycelial growth is retarded or whether the mycelium comes from the fluffly sector. Many abnormal unfused clamp cells were found in both mycelial areas of abnormal and normal appearance (**E** and **F**).

8.4.4. Ras ^{Val19} influences the fruiting body development

We found that "semi-*ras*^{Val19} dikaryons" such as 218/*ras*^{Val19} X PS001-1 and FA2222/*ras*^{Val19} X PS001-1 had defects in fruiting body development. After 2 days of culturing under fruiting conditions, primary and secondary hyphal knots were formed in abnormal numbers, ≥ 500 of primordia of a size of 0.05-0.1 mm developed in some YMG/T plates (Fig. 11 B), but they formed only a few 4 to 5 mature fruiting bodies per plate size of 0.5-15 mm. In contrast, control dikaryons (FA2222/pCc1001 X PS001-1, Fig. 11 A) formed in average 10 mature fruiting bodies per plate. These fruiting bodies had a size between 40-60 mm.

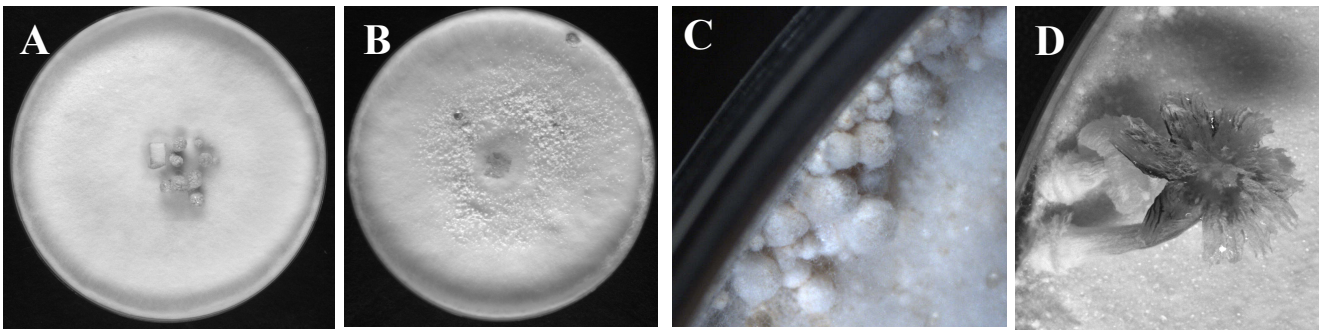


Figure 11. Comparison of the secondary hyphal knots (small condensed hyphal aggregates with yet undifferentiated tissues) and formation of primordial of between a control dikaryon FA222/pCc1001 X monokaryon PS001-1 (**A**) and semi-*ras*^{Val19} dikaryon FA2222/*ras*^{Val19} X PS001-1 (**B** and **C**). Note the abnormal high number of secondary hyphal knots (**B**) and of primordia (**C**) in culture of the semi-FA2222/*ras*^{Val19} X PS001-1 dikaryon and the small size of the mature fruiting body being while due to block in basidiospore formation (**D**).

From the size of mature fruiting bodies as well as from the global shape of the earlier primordium it appeared that *ras*^{Val19} might influence the general tissue structure of primordia and the fruiting bodies. Therefore, sectioning of primordia was preformed throughout development till the immature fruiting body, with structures from the semi- *ras*^{Val19} dikaryon 218/*ras*^{Val19} X PS001-1 and with structures from the control dikaryon 218/pCc1001X PS001-1.

The sections of primordia were observed under a binocular. It was found that the stipe and cap tissues of *ras* ^{Val19} primordia were altered (Fig 12). As a consequence of this, they formed a short stipe (size 0.5-20 mm long) and a small cap (5-10 mm diameter). Such tiny mushrooms occurred in culture of both dikaryons FA2222/*ras*^{Val19} X PS001-1(Fig. 11D) and 218/*ras*^{Val19} X PS001-1 (Fig. 12).

8.4.5. Ras ^{Val19} affects basidiospore formation and may influence the next generation

Fruiting bodies of dikaryon 218/*ras*^{Val19} X PS001-1 and FA2222/*ras*^{Val19} X PS001-1 carrying a *ras*^{Val19} allele were surprisingly white coloured or only light brown due to a repression of basidiospore formation (Fig. 11 and 12). Normal fruiting bodies are stained black by the colour of the mature basidiospores (see Fig. 12 and Fig. 13 A).

The features of basidiospores from *ras* ^{Val19} mushrooms (218/*ras*^{Val19} X PS001-1 and FA2222/*ras*^{Val19} X PS001-1) were then observed under the light microscope. There were various sizes of basidiospores i.e. big, medium and small sizes (Fig. 13 B and C), when compared with the wild type basidiospores, which have only one medium size (Fig. 13 A). About 50 % of the basidiospores of the fruiting body of semi-*ras*^{Val19} dikaryon FA2222/*ras*^{Val19} X PS001-1 have a middle size with the normal brown pigment. A large portion of spores of all three size types was not or only poorly stained (Fig. 13 B and C). Basidia of older primordia of dikaryon 218/*ras*^{Val19} X PS001-1 at the developmental stage after meiosis II were observed in the microscope (Fig. 14 A and C) and their nuclei stained. Basidia had four nuclei as to be expected after meiosis, suggesting that the failure in basidiospore formation in the dikaryon 218/*ras*^{Val19} X PS001-1 is not due to a defect in karyogamy or meiosis.

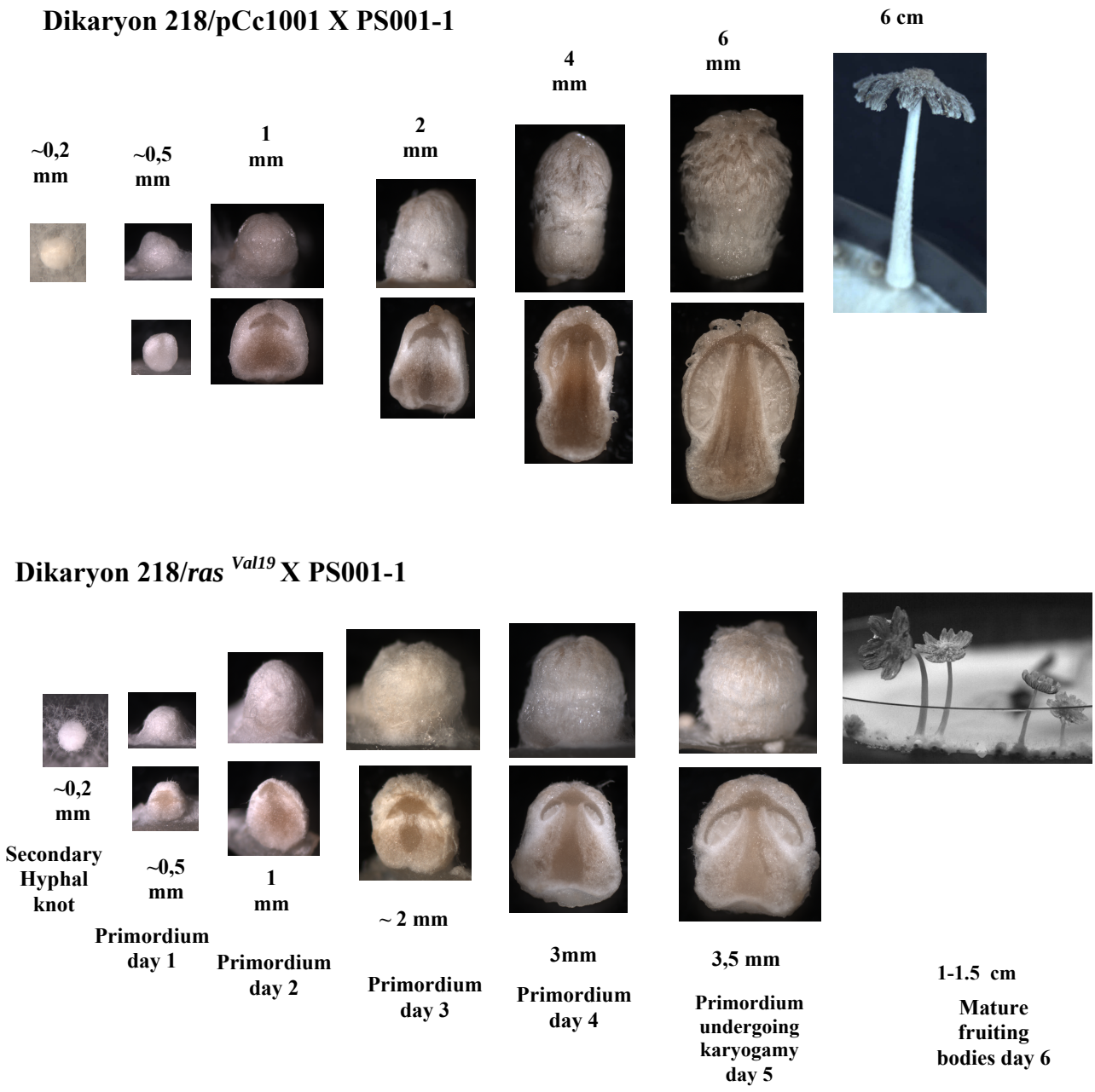


Figure 12. Primordia were harvested in mornings on day 1, day 2, day 3, day 4 and day 5 of development from a control dikaryon 218/pCc1001 X PS001-1 and a semi *ras*^{Val19} dikaryon 218/*ras*^{Val19} X PS001-1. At day 5 in the evening, primordia started to form the immature fruiting body. The mature fruiting bodies were formed after the midnight of day 5. At day 6, the wild type primordia showed the normal elongated stipes that increase in height till the end of mature fruiting body maturation, whereas the *ras* ^{Val19} primordia showed a short stipe with little elongation (Photos: courtesy of Monica Navarro-González).

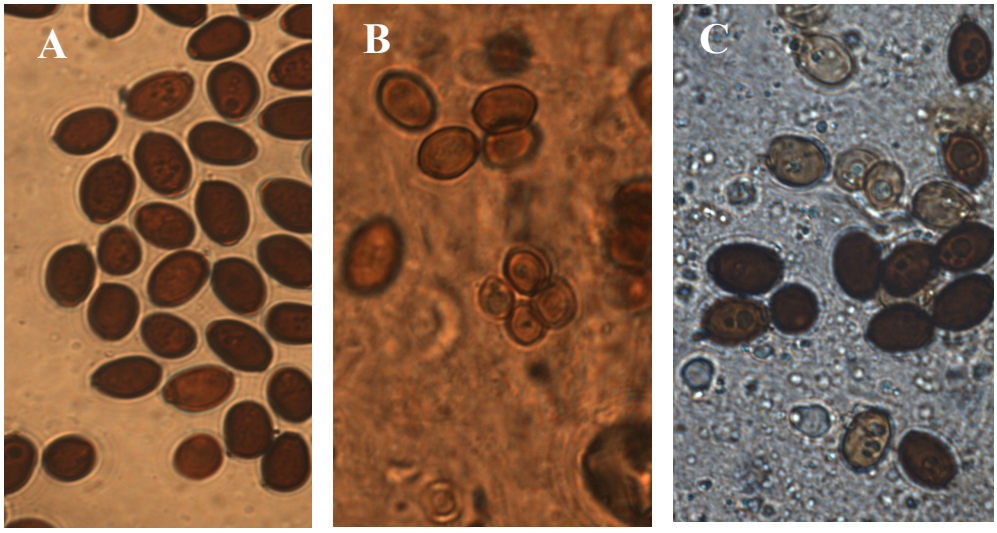


Figure 13. The basidiospores of a wild type mushroom from FA2222/pCc1001 X PS001-1 (**A**). The basidiospore of a *ras*^{Val19} mushroom from FA2222/*ras*^{Val19} X PS001-1 with the different sizes (big, middle and small sizes) (**B** and **C**).

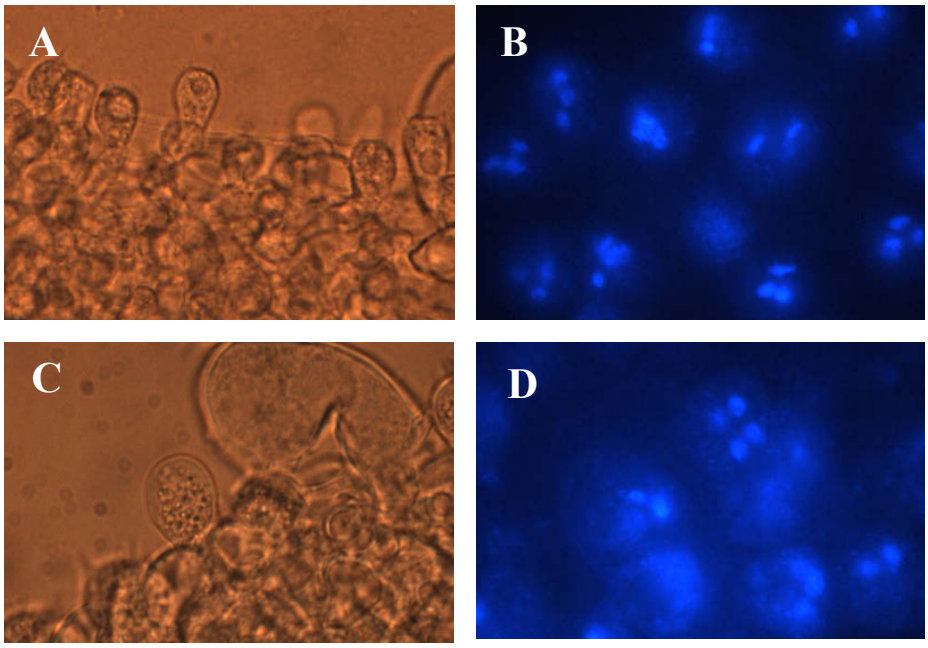


Figure 14. The basidia of a *ras*^{Val19} mushroom from dikaryon 218/*ras*^{Val19} X PS001-1 (**A** and **C**) and nuclei within the basidia after meiosis II stained by DAPI (**B** and **D**).

The basidiospores of *ras*^{Val19} mushrooms from cross 218/*ras*^{Val19} X PS001-1 and cross FA2222/*ras*^{Val19} X PS001-1 were then germinated on YMG/T plates and incubated at 37 °C for 36 hours. Two types of colonies appeared normal growing colonies and small dense (Fig. 15).

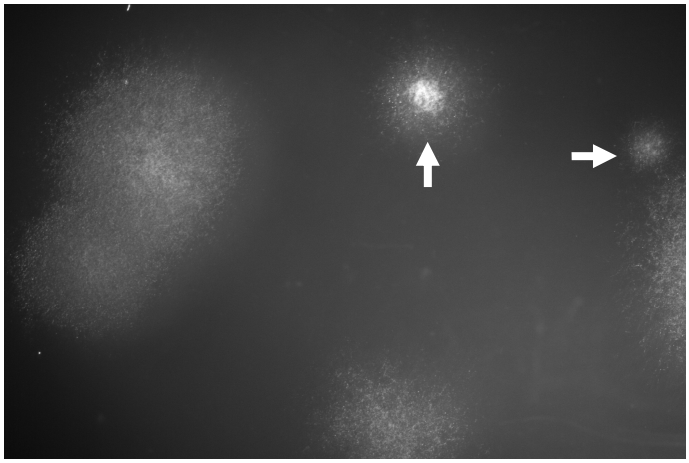


Figure 15. Spores of a *ras* mushroom of semi-*ras* ^{Val19} dikaryon FA2222/*ras*^{Val19} X PS001-1 germinated into colonies on YMG/T plates. The arrows point to s mall dense colonies.

For better expression of the colony growth phenotype, all single clones from YMG/T were transferred onto MM. After 36 hours, incubated at 37 °C, about 50% of the transferred colonies had a normal phenotype. 37-39% of transferred colonies had a dense small colony growth. 8-14% of the transferred colonies did not grow on minimal medium. The data suggest that very likely both the *trp1*$^{+}$ gene and the *ras*^{Val19} gene inserted during transformation with the chromosome with the *trp*$^{-}$ gene in both tested transformants and when in meiosis, there is some recombination between the chromosomes with the *trp*$^{-}$ copy from the transformants and the homologous chromosome with the *trp*$^{+}$ copy of monokaryon PS001-1 that give rise to the about 10% of *trp*$^{-}$ colonies in the progenies.

Table 5 Phenotype of normal and abnormal of basidiospores of *ras*^{Val} mushrooms on MM

Basidiospores of *ras*^{Val19}transformants	**Total clones**	**Not grown**	**Normal phenotype**	**Dense phenotype**
218/*ras*^{Val19} x PS001-1	84 (100%)	12 (14%)	41 (49%)	31 (37%)
FA2222/*ras*^{Val19} x PS001-1	95 (100%)	8 (8%)	50 (53%)	37 (39%)

8.5. Discussion

In this study, we transformed a cloned *ras* wild type gene (*ras*^{Wt}), a constitutively inactived *ras* allelel (*ras*^{Asn24}) and a constitutively activated *ras* allele (*ras*^{Val19}) into monokaryons of *C. cinerea*. Whilst no severe changes in phenotypes occurred with the *ras*^{Wt} and the *ras*^{Asn24} copy, introduction of *ras*^{Val19} had very severe effects. We observed that the Ras^{Val19} protein affected the colony growth including decrease of aerial mycelium and loss in hyphal growth direction in both, monokaryons and dikaryons. In some transformants (218 monokaryon), there were in addition small mycelial bodies in the aerial mycelium. Presence in the semi-*ras*^{Val19} dikaryon 218/*ras*^{Val19} X PS001-1 and the semi- *ras*^{Val19} FA2222/*ras*^{Val19} X PS001-1 furthermore caused abnormal clamp cell phenotypes: clamps at the same hyphae grow in different directions, some septa had more than one clamp cell and some clamp cells showed prolonged tip growth. Fruiting bodies in the semi-*ras*^{Val19} dikaryon 218/*ras*^{Val19} X PS001-1 and the semi-*ras*^{Val19} dikaryon FA2222/*ras*^{Val19} X PS001-1 were small, had an altered tissues distribution and no or only a few spores.

These results were consistent with previous reports on fungal transformants of activated *ras* genes and of *gap*-knockout strains that conserve the active GTP-bound *ras* form within the cells, implying that the Ras protein is a central regulator of developmental processes in fungi. In filamentous ascomycetes, phenotypes of curly growing hyphae with many short branches, invasive hyphal growth and a reduction of aerial mycelium have been reported (Fortwendel et al. 2005; Thevelein et al. 1999). In other cases, asexual sporulation was suppressed (Truesdell et al. 1999; Toda et al. 1985) but this was not true for *C. cinerea*. Thus, in between different fungi alike phenotypes but also different phenotypes might be controlled through *ras*.

In *S. cerevisiae,* Ras signaling causes an increase of intracellular cAMP levels, and cAMP is known to regulate cell proliferation and carbon metabolism (Thevelein and Winde 1999). Changes in hyphal growth direction in filamentous of fungi have been explained by a disturbance nutrient sensing (Bottoli 2001). Nutrient starvation in various fungi is sensored *via* cAMP (Kronstad et al. 1998; Alpaugh et al. 2000).

In *C. cinerea*, cAMP is known to be needed for fruiting body production which is initiated in development once the nutrients are depleted (Uno et al. 1974; Kües et al. 2004). Here, we see an effect of the activated *ras*^{Val19}allele on fruiting body production. We have many more secondary hyphal knots and primordia as normally occur. Thus, one might expect that through *ras*^{Val19} activation, more cAMP is produced in the mycelium with the consequence of higher frequency of fruiting body initiation. cAMP is also known to be increased at later stages of fruiting. The peak in cAMP corresponds to 2 mm sized primordia at day 3 of mushroom development (Kües et al. 2004; Kronstad et al. 1998) which corresponds to the stage where we saw alteration of tissues formation

in the primordia (Fig. 12). Mushrooms on semi-*ras*^{Val19} dikaryons 218/*ras*^{Val19} X PS001-1 are smaller than normal. In normal development, chemical communication between cap and stipe tissue have to occur in order to elongate stipes and expand the caps (Kües 2000). It is possible that the small size of mushrooms in the semi-*ras*^{Val19} dikaryon 218/*ras*^{Val19} X PS001-1 may link to disturbance of this chemical communication due to the altered tissue structure. Another phenotype linked to the fruiting bodies is the lack of basidiospores. If some are formed, they are partially altered in size and/or in pigmentation. Microscopy of nuclei in basidia after meiosis revealed that in basidia there is not a defect in karyogamy or meiosis that is responsible for this effect but an unknown post-meiotic event.

A previously study on mating type genes in *C. cinerea* showed that the *B* mating type genes are required at the stage of karyogamy (Kües et al. 1989, 2002) in order to induce stipe elongation and cap expansion. However, the block in development here is after the *B* mating type induced steps in fruiting body development. During the first step in fruiting, the induction of secondary hyphal knots, the *B* mating type genes support the *A* mating type genes in frequency of fruiting body initiation (Kües et al. 2002). However, at this point of development obviously both the Ras signaling (this study) and the *B* mating type pathway are involved. Clamp cell fusion is another phenotype controlled by the *B* mating type genes (Day 1969) and, as shown here, by the Ras signaling pathway.

Our results showed that the tip of abnormal unfused clamp cells continued to grow and most of the clamp cell tips grew over the pegs formed sup-apical to the foremost septum (Badalyan 2004; Schubert et al. 2006). Consequently, clamp cell fusion failed, probably because the signal that makes the clamp cells to recognize the peg sub-apical cells was lost. Thus, the signal might be the *B* mating type pheromones (Kothe 1996; Brown and Casselton 2001).

Schubert et al. (2006) reported that there were many abnormal clamp cells in a dikaryon of *S. commune* carrying no *Gap1* gene with the result that tips of the clamp cell continued to grow beyond the peg at the sub-apical cell and that they missed fusion. Also the unusual behavior of the tip of the clamp cell in the *C. cinerea* dikaryons and in the *S. commune Δgap* dikaryons could be a problem of distributed nutritional sensing.

8.6. References

Alspaugh, J. A., L. M. Cavallo, J. R. Perfect and J. Heitman, 2000 RAS 1 regulates filamentation, mating and growth at high temperature of *Crytococcus neoformans*. Mol. Microbiol. **36:** 3206-3217.

Alpaugh, J. A., R. Pukkila-Worly, T. Harashima, L. M. Cavallo, D. Funnell *et al.*, 2002 Adenylyl cyclase functions downsteam of the G protein Gpa1 and controls mating and phathogenicity of *Cryptococcus neoformans*. Eukaryot. Cell **1:** 75-84.

Bivona, T. G., and M. R. Philips, 2003 Ras pathway signaling on endomembranes. Curr. Opin. Cell Biol. **15:** 136-142.

Boguski, M. S., and F. MecCormick, 1993 Proteins regulating Ras and its relatives. Nature **366:** 643-54.

Bottoli, F. P. A., 2001 *Metabolic and Environmental Control of Development in Coprinus cinereus*. Ph.D thesis, Swiss Federal Institute of Technology Zurich, Switzerland.

Downward, J., 2003 Targeting Ras signaling pathways in cancer therapy. Rev. Cancer **3:** 11-22.

Dürrenberger, F., K. Wong, and J. W. Kronstad, 1998 Identification of the cAMP-dependent protein kinase catalytic subunit required for virulence and morphgenesis in *Ustilago maydis*. Proc. Natl. Acad. Sci. USA **95:** 5684-5689.

Farnsworth, C. L., and L. A. Feig, 1991 Dominant inhibitory mutations in the Mg^{2+} binding site of RasH prevent its activation by GTP. Mol. Cell Biol. **11:** 4822-4829.

Fortwendel, R. J., W. Zhao, R. Bhabhra, S. Park, D. Perlin *et al.*, 2005 A fungus-specific *ras* homolog contributes to the hyphal growth and virulence of *Aspergillus fumigatus*. Eukaryot Cell **4:** 1982-1982.

Ishibashi, O., and K. Shishido, 1993 Nucletotide sequence of a ras gene from the basidiomycete *Coprinus cinereus*. Gene **125:** 233-234.

Kertesz-Chaloupková, K., P. J. Walser, J. D. Granado, M. Aebi, and U. Kües, 1998 Blue light overrides repression of asexual sporulation by mating type genes in the basidiomycete *Coprinus cinereus*. Fungal Genet. Biol. **23:** 95-109.

Kronstad, J., A. D. Maria, D. Funnell, R. D. Laidlaw, N. Lee *et al.*, 1998 Signaling via cAMP in fungi: interconnections with mitogen-activated kinase pathways. Arch. Microbiol. **170:** 395-404.

Kües, U., 2000 Life history and developmental processes in the basidiomycete *Coprinus cinereus*. Micro. Mol. Biol. Rev. **64:** 316-353.

Kües, U., J. D. Granado, R. Hermann, R. P. Boulianne, K. Kertesz-Chaloupková *et al.*, 1998 The *A* mating type and blue light regulate all known differentiation processes in the basidiomycete *Coprinus cinereus*. Mol. Gen. Genet. **260:** 81-91.

Kües, U., M. Künzler, A. P. F. Bottoli, P. J. Walser, J. D. Granado *et al.*, 2004 Mushroom development in higher basidiomycetes; implications for human and animal health, pp. 431-470 in *Fungi in Human and Animal Health*, edited by R. K. S. Kushwaha, Scientific Publishers, Jodhpur, India.

Kües, U., P. J. Walser, M. J. Klaus, and M. Aebi, 2002 Influence of activated *A* and *B* mating-type pathways on developmental processes in the basidiomycete *Coprinus cinereus*. Mol. Genet. Genomics **268:** 262-271.

Matthews, T. R., and D. J. Niederpruem, 1972 Differentiation in *Coprinus lagopus*. I. Control of fruiting and cytology of initial events. Arch. Mikrobiol. **87:** 257-268.

O'Shea, S. F., P. T. Chaure, J. R. Halsall, N. S. Olesnicky, Matthews, T. R. 1998 A large pheromone and receptor gene complex determines multiple *B* mating type specificities in *Coprinus cinereus*. Genetics **148:** 1081-1090.

Polak, E., R. Hermann, U. Kües, and M. Aebi, 1997 Asexual sporulation in *Coprinus cinereus*: structure and development of oidiaphores and oidia in an AmutBmut Homokaryon. Fungal Genet. Biol. **22:** 112-126.

Raper, J. R., and R. M. Hoffman, 1974 *Schizophllum commune*, pp.597-626 in *Handbook of Genetics*, Vol I., edited by R. C. King. Plenum Press, Inc., New York.

Regenfelder, E., T. Spelling, A. Hartmann, S. Lauenstein, M. Bolker *et al.*, 1997 G-proteins in *Ustilago maydis*: transmission of multiple signals. EMBO J. **16:** 1934-1942.

Schubert, D., M. Raudaskoski, N. Knabe, and E. Kothe, 2006 Ras GTPase-activating protein gap1 of the homobasidiomycete *Schizophyllum commune* regulates hyphal growth orientation and sexual development. Eukaryot. Cell **5:** 683-695.

Toda, T., I. Uno, T. Ishikawa, S. Power, T. Kataoka, *et al.*, 1985 In yeast, Ras proteins are controlling elements of adenylate cyclase. Cell **40:** 27-36.

Thevelein, J. M., and J. H. D. E. Winde, 1999 Novel sensing mechanisms and targets for the cAMP-protein kninase A pathway in the yeast *Saccharomyces cerevisiae*. Mol. Microbiol. **33:** 904-918.

Uno, I., and T. Ishikawa, 1971 Chemical and genetic control of induction of monokaryontic fruiting bodies in *Coprinus macrorhizus*. Mol. Gen. Genet. **113:** 228-239.

Uno, I., and T. Ishikawa, 1973 Metabolism of adenosine 3',5'-cyclic monophosphate and induction of fruiting bodies in *Coprinus macrorhizus*. J. Bacteriol. **113:** 1249-1255.

Wang, P., C. B. Nichols, K. M. Lengeler, M. E. Cardenas, G. M. Cox *et al.*, 2002 Mating type specific and nonspecific PAK kinases play shared and divergent roles in *Cryptococcus neoformans*. Eukaryotic Cell **1:** 257-272.

Wang, Y., M. Boguski, M. Riggs, L. Rodgers, and M. Wiggler, 1991 *Sar1*, a gene from *Schizosaccharomyces pombe* encoding a protein that regulates ras1. Cell Regul. **2:** 453-465.

Wessels, J. G. H., 1978 Incompatibility factors and the control of biochemical processes, pp. 81-104 in *Genetics and Morphogenesis in the Basidiomycetes*, edited by M. N. Miles and P. G. Miles. Academic Press, Inc., New York.

CHAPTER 9

Expression of the homeodomain transcription factor encoding mating type genes from foreign species in *Coprinopsis cinerea* and test of functioning of foreign promoters from genes that are controlled in expression in the fungal host by the homeodomain transcription factor mating type pathways

9.1. Abstract

Sexual development of the higher basidiomycetes *Coprinopsis cinerea*, *Ustilago maydis* and *Schizophyllum commune* is regulated by mating type genes. These three basidiomycetes are tetrapolar and have two mating type loci, namely the *A* and the *B* mating type loci in the two higher basidiomycetes, and the *b* and the *a* mating type loci in *U. maydis*. The *A* mating type loci of *C. cinerea* and *S. commune*, and the corresponding *b* mating type locus of *U. maydis* consists of genes that encode two types of homeodomain transcription factors (HD1 and HD2). For a successful mating reaction, an HD1 protein and an HD2 protein of different mating type specificities (coming from compatible strains) have to interact. Here, we present studies on the expression of homeodomain transcription factors encoding mating type genes of two foreign species in *C. cinerea.* In one positive experiment, the rate of transformation efficiency of functional expression of *U. maydis* homeodomain transcription factor genes was between 12-19 %, and of *S. commune* homeodomain transcription factor genes was between 10-30 % of transformants. Expression of clamp cells was only transiently and only in certain sectors of colonies of transformants. However, in two repeats of the experiments, no positive clones were observed upon transfer of transformants from regeneration agar to solid minimal medium, also not when changing the growth temperatures.

In this study, we further observed the functions of foreign promoters i.e., *Sc3* and *Sc4* from *S. commune*, and *gdpII* from *A. bisporus* in monokaryons, dikaryons, and in fruiting body development of *C. cinerea*. To test the promoter function, we used the laccase gene (*lcc1*) of *C. cinerea* with an ABTS oxidation test. All three promoters were found to be active in both hyphae of mycelia and fruiting bodies. In vegetative mycelia the *gdpII* promoter was the best, whereas in the fruiting structures the *Sc4* promoter was more active than the *Sc3* promoter. In dikaryons formed by mating of transformations of two compatible strains, combination of the same gene promoters *gdpII/gdpII* and *Sc3/Sc3* gave highest expression. Although the studies presented gave a number of interesting observations, the present results can only be considered as preliminary. In the future, much more extensive research has to be carried out to follow up the phenomena seen in this study.

9.2. Introduction

Coprinopsis cinerea, *Schizophyllum commune* and *Ustilago maydis* belong to the basidiomycetes. These species are typical models for studying the fungal development, including fruiting body development in case of the higher basidiomycetes. In terms of ecology, the three species are representatives of the saprophytic fungi (*C. cinerea*), of wood decay fungi (*S. commune*), and of plant fungi (*U. maydis*), respectively. Sexual reproduction in the life cycle of these fungi is regulated by the mating type genes (Kronstad and Staben 1997; Kües 2000; Kothe 2001). These basidiomycetes are tetrapolar and have two mating type loci, which are located on the different chromosomes (Raper 1966; Kronstad and Staben 1997; Muraguchi et al. 2003).

One of these mating type loci of the basidiomycetes is similar to the single mating type locus (*Mat*) of the ascomycete yeast *Saccharomyces cerevisiae* (Kües and Casselton 1992, 1993). This ascomycete was studied and found to encode a homeodomain transcription factor in each of the two alleles of the *Mat* locus (***a1*** and α*2*). These homeodomain transcription factors have to interact as an **a**-1/**α**-2 heterodimer to regulate sexual development upon mating (Herskowitz et al. 1992).

The mating type locus of the basidiomycetes that is similar to *Mat* locus of *S. cerevisiae* also encodes two different types of homeodomain transcription factors (HD1 and HD2). Thereby, the HD1 transcription factors are homologous to the **α**-protein of *S. cerevisia*e, and the HD2 transcription factors are homologous to the **a** transcription factor. As in yeast, in basidiomycetes an HD1 protein has to interact with an HD2 protein to form an active transcription factor complex that can regulate sexual development after mating. As a difference to yeast, however, the basidiomycete mating type locus has multiple alleles. To ensure that every allele of the mating type locus in a basidiomycetes can interact with a different allele from the same species, both types of homeodomain transcription factors genes reside in pairs in the mating type locus. HD1 and HD2 proteins are coming from the same gene pairs can not interact, but HD1 and HD2 proteins from allelic gene pairs can interact (Kües and Caselton 1992, 1993). The genes in the *A* mating type locus of *S. commune* are called *Z* and *Y* or *HD1* and *HD2* genes, respectively (Robertson et al. 1996), consisting of many alleles named *Y1*, *Y2*, *Y3* and *Y4*…, and *Z2*, *Z3* and *Y4* (a *Z1* allele is not existing) …, respectively. An active heterodimer between *Y3* and *Z4* is formed. In *U. maydis*, the *b* mating type locus consists of multiallelic genes named *bE* and *bW*, and alleles are named *bE1, bE2, bE3…* and *bW1, bW2, bW1…,* respectively. An active heterodimer is formed for example between bE1 and bW2.

In this study, we test in gene transformation a single mating type protein from *S. commune* and *U. maydis* can also be active with native proteins in *C. cinerea*. As a test system for gene activity and for successful heterodimer formation, clamp cell production has been shown before to be activated in *C. cinerea* transformants of monokaryons upon introduction of *HD1* or *HD2* genes of different mating type specificity (Kües et al. 1992; Mutasa et al. 1990). In the structurally different vegetative mating of the monokaryons and of the dikaryon as well as in the fruiting bodies of the higher basidiomycetes, different sets of genes are expected to be expressed, some of which that might be under direct or indirect positive control of the HD1-HD2 mating type transcription factors complex, and some of which that might be under direct or indirect negative control. An important class of genes shown to be under such a mating type gene control are the hydrophobin genes (Wessels 1994; Velagapudi 2006).

Hydrophobins are small proteins of 100-140 amino acid sequence in length that are very dissimilar in sequence with the exception of 8 conserved cysteine residues. Upon secretion, hydrophobins self-assemble into amphipathic stable films that cover fungal cells and make their surfaces hydrophobic. These hydrophobic films covering the hyphae enable the fungal structures to grow into the air and protect the fungal cells from adverse environmental conditions (Velagapudi, unpublished; Wösten 2001). *S. commune* has at least 4 hydrophobin genes that are called *SC1*, *SC3*, *SC4* and *SC6* (Wessels et al. 1995), whereas *C. cinerea* has the impressing number of 34 different hydrophobin genes (Velagapudi 2006). In *S. commune,* the *SC3* gene is active in both the monokaryons and the dikaryons. In contrast, the *SC4* gene promoter is active in the dikaryon and quite reasonably during fruiting body development. The *SC4* gene is expressed by the submerged hyphae of the vegetative dikarotic mycelia and the hyphae in the inner tissue of the fruiting bodies with a highest expression at the primordial stage (Mulder and Wessels 1986). Expression of the *SC4* gene in the fruiting body is high when compared to the other two genes *SC1* and *SC6*. Van Wetter and co-workers further studied the expression of *SC4* gene by putting it under the control of the promoter of the *SC3* gene. Thereby, the combined promoter is active in both the monokaryons and the dikaryons. Therefore, the *SC3* promoter can also be used for high-level gene expression of the other genes (Wessels et al. 1987). The *SC3* promoter of *S. commune* has also been shown to be active in the expression of a laccase gene in *Pycnoporus cinnabarinus* (Alves et al. 2004) and in *C. cinerea* (Kilaru et al. 2006).

Laccase reacts with the ABTS (2, 2´-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid), a colourless compound that turns into a green-blue colored product upon the enzymatic oxidation. Photometric tests of laccase activities can be performed to determine amounts of enzymes secreted by fungi into media (Dwivedi 2006). Color reactions also occur when ABTS is added to solid medium on which fungi grow that secrete laccase. Depending on the type of medium, the agar stains green-blue (minimal medium) or red-brownish (YMG/T complete medium). With increasing enzyme production, agar stains underneath fungal colonies, or stained halos are formed around fungal colonies whose width correlates with the amounts of enzyme produced. Therefore, the cloned laccase gene *lcc1* from *C. cinerea* can be used as a reporter gene for the promoter activity in *C. cinerea* (Kilaru 2006; Kilaru et al. 2006).

Kilaru and co-worker used the developmentally regulated *Sc3* and *Sc4* promoters of *S. commune*, and the constitutively active *gdpII* promoter of *Agaricus bisporus* (from the glyceraldehyde-3-phosphate dehydrogenase gene) and joined them upstream to the laccase gene *lcc1* of *C. cinerea* in order to increase the amount of laccase enzyme production in this species during the vegetative growth (Kilaru 2006; Kilaru et al. 2006).

9.3. Material and methods

9.3.1. Expression of heterologous *A* mating type genes in *C. cinerea*

Coprinopsis cinerea monokaryons strain 218 (*A3 B1 trp1.1, 1.6, bad*) and strain FA2222 (*A5 B6, acu-1, trp1.1, 1.6*) (Kertesz-Chaloupková et al. 1998) were used as the fungal hosts to analyze the interaction between the mating type genes of *C. cinerea* and the foreign mating type genes. pCc1001 carrying the complete tryptophan synthase gene (*trp1+*) was used as the selective plasmid for co-transformations (Binninger et al. 1987). pZ4 and pY3 carrying the *A* mating type genes *Z4* and *Y3* of *S. commune*, respectively, and pbE1 and pbW2 carrying the *b* mating type genes *bE1* and *bW2* of *U. maydis*, respectively, were used in this study. These plasmids were kindly provided by R. Ullrich and R. Kahman from the Universities of Vermont and Marburg, respectively. DNA transformation was performed as previously described by Granado et al. (1997).

9.3.2. Expression of heterologous *S. commune* promoters (*Sc3* and *Sc4*) in *C. cinerea*

1 μg of plasmid pCc1001 for selection (Bininger et al., 1987) was co-transformed into *C. cinerea* monokaryons 218 (*A3 B1 trp1.1,1.6, bad*) and FA2222 (*A5 B6, acu-1, trp1.1,1.6*) strains with 1 μg either of pYSK7, pYSK17 or pYSK39 (Kilaru et al. 2006). These constructs were kindly obtained from Sreedhar Kilaru. They are all identical, but the promoter sequence inserted upstream to the laccase gene (*lcc1*) gene of *C. cinerea*. pYSK7 contains the *gdpII* promoter from *Agaricus*

bisporus, and pYSK17 contains the *S. commune Sc3* promoter, whereas pYSK39 contains the *S. commune Sc4* promoter (Kilaru et al. 2006; Kilaru, unpublished). Transformed protoplasts of these construct were plated on regeneration medium (Granado et al. 1997) containing 0.5 mM ABTS (2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid). Transformants were picked and further grown at room temperature on minimal medium (MM). Selected transformants were then inoculated and grown on MM and YMG (Yeast-Malt-Glucose) agar medium supplemented with 0.5 mM of ABTS. The positive laccase transformants produced a green-color on MM, and a brown-color on YMG due to the oxidation of ABTS with laccase enzyme. Laccase/218 transformants were crossed with laccase/FA2222 transformants on YMG agar plates to produce laccase/dikaryons.

To check the enzymatic activities, the clones and dikaryons of transformants were grown in 500 ml Erlenmeyer flask containing 100 ml of YMG liquid medium at 37°C in the incubation shaker with the 120 rpm. In case of dikaryon, 0.1 mM of $CuSO_4$ was added to the 100 ml cultures. Cultures were inoculated with 5 ml of a mycelium suspension obtained from a homogenate of a 6 day-old YMG agar pre-culture in 50 ml of sterile YMG medium. Enzyme activities were checked in liquid shaken cultures over the time, each day 0.5 ml of culture supernatants was taken. The mycelial particles were separated by centrifugation for 5 min at 13,000 rpm. Laccase activity was determined at room temperature by monitoring the oxidation of ABTS at 420 nm in 120 mM of sodium acetate buffer (pH 5.0) (Matsumura et al. 1986), using 20 µl of supernatant sample and 20 µl of 0.5 mM ABTS in a final volume of 200 µl. One unit of enzyme activity (IU) represents the amount of laccase that oxidizes 1 µmol substrate/min.

9.3.3. Test for the activity of heterologous promoters in *C. cinerea*

For fruiting, dikaryons were transferred into standard fruiting conditions (25°C, 12 hours/light and 12 hours/dark, and 80-90% of humidity; Granado et al. 1997). The primordia of different ages, immature and mature fruiting bodies were harvested. The cap and stipe tissues were separated, and then each tissue was grinded by using a mortar and pestle. The homogenized tissue was then transferred into 1.5 ml Eppendorf tubes containing 1 ml of 120 mM sodium acetate buffer (pH 5.0), vigorously mixed by using a Vortex mixer, kept on ice for 30 min, and then centrifuged at 13,000 rpm, at 4°C, for 5 min. After that, 100 ml of supernatants were transferred into the new 1.5 ml Eppendorf tubes. The enzyme activities and supernatants were tested by the ABTS assay (Matsumura et al. 1986). Briefly, 100 µl of ABTS (0.5 mM) was added into 900 ml of supernatants to test the laccase activity in parallel with ABTS assay, and the mixture was then kept on the ice box to observe the laccase activity.

9.4. Results and Discussion

9.4.1. Expression of homeodomain transcription factors encoding mating type genes from heterologous species in *C. cinerea*

We transformed *A* mating type genes of *S. commune* and *b* mating type genes of *U. maydis* into *C. cinerea* monokaryons strains 218 and FA2222, and then observed clamp cell formation under the light microscope. In the different experiments performed with monokaryon FA2222, we never observed any transformants that produced clamp cells, although between 30 and 40 transformants were tested (Table 1). For comparison, co-transformation rates of clamp cell production in transformation of homologous *A* mating type genes in *C. cinerea* in experiments with other strains were between 29 and 39 % (Kües et al. 2001).

Table 1 Number of FA2222 clones analyzed after transformation with the homeodomain transcription factor encoding mating type genes from foreign species

Experiment	pCc1001 control	*U. maydis*		*S. commune*	
		pbE1	***pbW2***	***pZ3***	***pY4***
1	30	40	40	40	40
2	30	40	40	40	40
3	40	40	40	40	40

In contrast, in one of three performed experiments when mating type genes of *U. maydis bE1* and *bW2* were transformed into *C. cinerea* monokaryon 218, functional expression of clamp cell formation was found in the rates between 12-19 % of the transformants. When the *S. commune* genes *pY3* and *pZ4* were transformed into *C. cinerea* monokaryon 218, functional expression of clamp cells was found in the rates between 10-30 % (Table 2). However, we found clamp cell formation not throughout the whole colonies of transformants of *C. cinerea* strain 218, but in sectors with loose growing mycelia (Fig. 1). The mycelium sectors grew faster than the other parts of the colonies as a typical characteristic for the transformants with an activated *A* mating type pathway (compare with chapter 5 of this thesis).

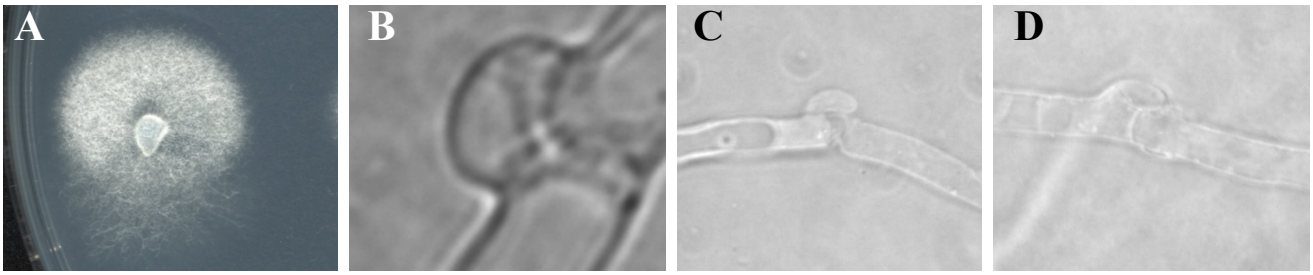


Figure 1. Clones of a first transformation experiment of monokaryon 218 with homeodomain transcription factors encoding mating type genes. Clones were picked from regeneration agar onto MM plates, grown at 37°C in the dark before observation of hyphae under the microscope (**A),** Mycelial sectors with a colony of a bW2 transformant of monokaryon 218. (**B),** unfused clamp cell on a hyphal septum of the same transformant. (**C)** and (**D)**, unfused clamp cells of 218 transformants of *S. commune Y3* and *Z4* genes, respectively.

Table 2 Percentage of 218 transformants expressing clamp cells in co-transformation with *U. maydis* and *S. commune* mating type genes with plasmid pCc1001

***A* mating type gene**	**Total number of clones [(a)] tested**	**Clones with clamp cell formation**	**Percentage of clones with clamp cell formation**
bE1	50	6	12%
bW2	36	7	19%
Y3	40	12	30%
Z4	10	1	10%
pCc1001 (control) *trp1+*	30	-	-

[a] In two other transformation experiments with monokaryon 218, 30 and 40 clones of the *bE1* transformation, 30 and 40 clones of *bW2* transformation, 40 and 40 clones of *pY3* transformation, and 40 and 40 clones of *pY4* transformation were tested on minimal medium where clamp cells were not observed.

In the two repeats of the 218 transformation experiments, microscoping hyphae of about 1-3 selected transformants from regeneration agar (selection criteria: loose mycelium growth of a colony with strong hyphae (Fig. 2), detected clamp cells on clones of all four mating type genes (Fig. 3). However, upon picking and growth at 37 °C, these clones and 30-40 further clones per transformation onto minimal medium, no clamp cell formation was defected on where mycelial sectors of loose hyphal growth seen. Transferring small pieces of mycelium onto new medium and growth at different temperatures (25 °C, 28 °C and 37 °C) did also not lead to an observation of clamp cell formation.

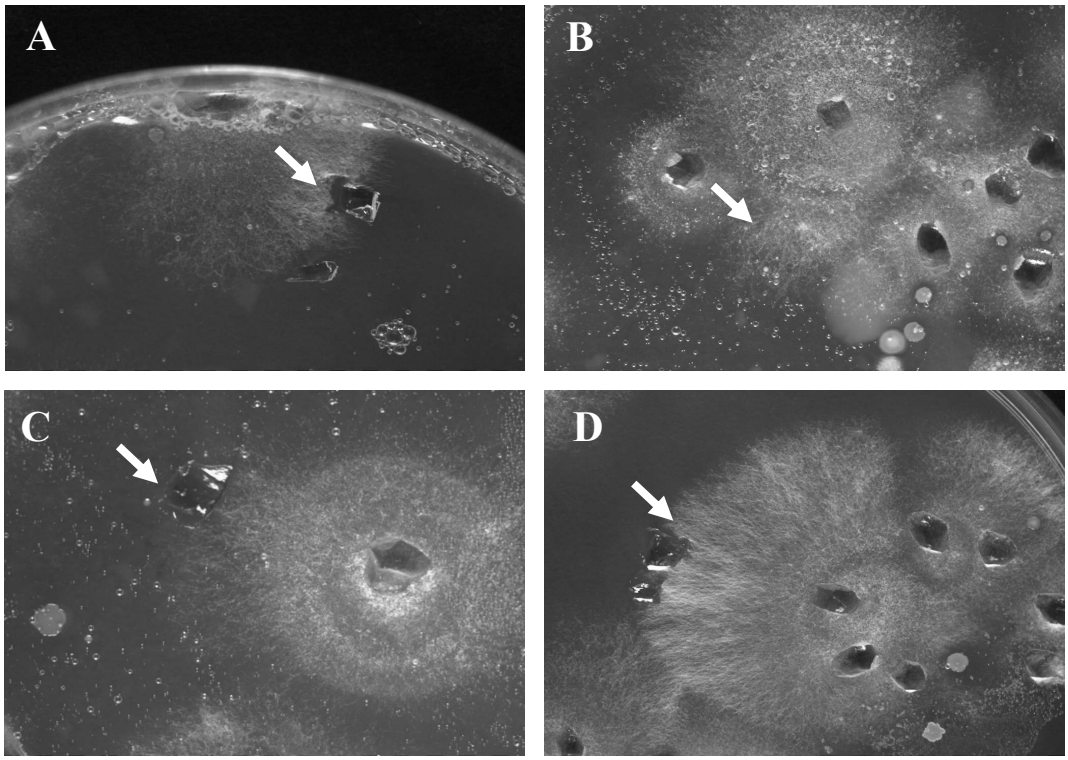


Figure 2. Clones of *C. cinerea* strain 218 after co-transformation of pCc1001 with *pW2* (**A),** *pE1* (**B**), *pY3* (**C**), and *pZ4* (**D**). The arrow heads point to the strong growth of hyphae found to contain clamp cells. (Note: transformants on regeneration agar from the second repeat of the experiment shown in Fig. 1). The plates shown in Figure 2 (B), (C) and (D) contain a few colonies of contaminating yeast. This might be occurred due to the complex handling of the oidia harvesting from a monokaryotic mycelia culture, protoplasting and protoplast transformation (Granado et al. 1997). Such contaminations occur occasionally in transformation experiments and are difficult to avoid.

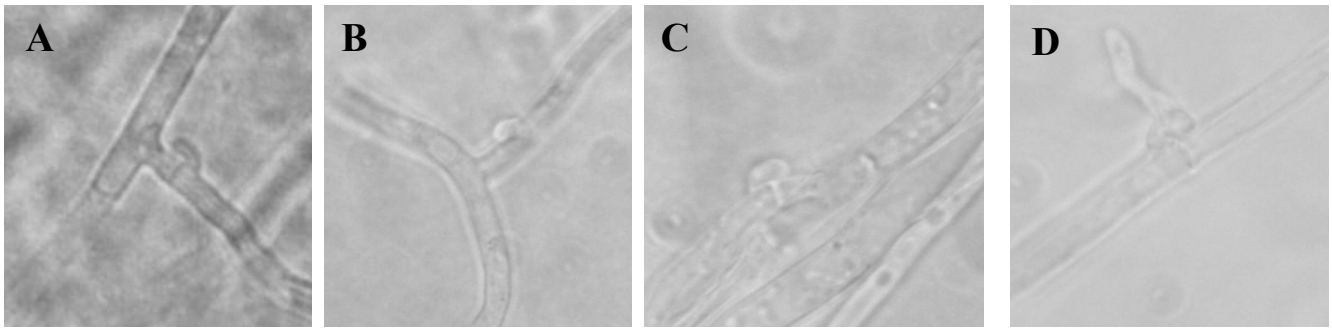


Figure 3. Clamp cell on hyphae of 218 transformants grown on regeneration agar, **A** of a transformant of *pW2*, **B** of a transformant of *pE1,* **C** of a transformant of *pY3*, and **D** of a transformant of *pZ4* (from the same experiment as shown in Fig. 2).

At the current stage, there is no satisfying explanation for the origin of the different behaviour of transformants in this experiments performed. Transient expression of clamp cell formation upon transformation of *S. commune A* mating type genes into *C. cinerea* monokaryons have been observed in previous experiments performed by U. Kües (personal communication). In her experiments, transformants were first incubated at 30°C in the dark when clamp cells were seen and then further at room temperature in the laboratory with day and night shifts when no clamp cells were produced. Whether environmental signal triggers the expression of clamp cells in the transformants, respectively block clamp formation is also not clear from her experiments.

In the literature, there are reports on some other experiments on expression of homeodomain transcription factors encoding mating type genes in heterologous species. Transformation of the genes from *Ustilago hordei* in *U. maydis* showed that both the *bE* and the *bW* homeodomain transcription factor of *U. maydis* can interact with the respective homeodomain transcription factors of *U. hordei* (Bakkeren and Kronstad 1993, 1996). More than that, the *A* mating type genes of *Coprinopsis scobicola* can activate clamp cell formation in *C. cinerea* (Kües et al. 2001), while the *A* mating type genes of *C. cinerea* can activate clamp cell formation in *C. scobicola* (Challen et al. 1993). However, only one type of HD1-HD2 protein combination between the two species was shown to be active, an *HD1* gene from *C. scobicola* in combination with an *HD2* gene of *C. cinerea* (Challen et al. 1993; Kües et al. 2001). Possibly the protein interaction required to form an active transcription factor complex is not possible between an HD2 protein of *C. scobicola* and an HD2 protein of *C. cinerea* or such interaction between the proteins might be so weak that clamp cell phenotypes in *C. cinerea* can not be achieved. In contrast, the *A* mating type genes of *Coprinellus disseminatus* can induce clamp cells formation in *C. cinerea* (James et al. 2006). The available results with the *C. disseminatus* genes however do not allow a discrimination between the function of *HD1* and *HD2* genes in *C. cinerea* since whole *A* mating type loci have been transformed and no individual genes (see chapter 7, James et al. 2006). From the reports in literature, it might therefore not be seen as a surprise to observe clamp cell formation in *C. cinerea* activated by heterologous mating type genes from *U. maydis* and *S. commune.* One might conclude from the experiments that the heterologous mating type proteins are able to interact with the resident mating type proteins of *C. cinerea* (Table 2). Instabilities of phenotypes such as seen in this study have however not been reported from the expression experiments published in the literature. Temperature effects were suspected to be one reason for such instability. However, the experiments performed in this work did not support this hypothesis. Light has been reported to affect the structure of *A* mating type controlled phenotypes in *C. cinerea* (Kertesz-Chaloupková et al. 1998; Kües et al. 1998). Further studies therefore might concentrate on testing the light effects on transformants in combination with the temperature changes.

9.4.2. Functional test of the *S. commune Sc3* and *Sc4* promoters in *C. cinerea* dikaryon

In order to test the activity of the *Sc3* and *Sc4* promoters of *S. commune* in the heterologous host *C. cinerea,* we transformed the *lcc1* plasmids pYSK17 with the *Sc3* promoter and pYSK39 with the *Sc4* promoter into the *trp1*-auxotrophic monokaryons FA2222 and 218 together with the plasmid pCc1001 carrying the *trp1*-wild type gene as selection marker. As the positive control for the laccase test, in a parallel transformation pYSK7 with *lcc1* under control of the constitutive *gpdII* promoter was introduced into the two monokaryons together with plasmid pCc1001. As a negative control, only pCc1001 was transformed.

FA2222 is a monokaryon without intrinsic laccase activity (Kilaru et al. 2006). Therefore, all green stained colonies obtained on regeneration agar are positive laccase transformants and could easily be selected. Picked FA2222 transformants (between 40-70 clones per transformation) were transferred onto solid YMG medium with 0.5 mM ABTS. After 2 days of growth at room temperature, observed halos around colonies were divided into three categories (+, ++, +++). Category + corresponds to halos in 0.1-0.2 cm width, category ++ to halos of a width of 0.2-0.3 cm, and category +++ to halos of 0.4-0.5 cm, and category – denotes colonies without staining around the colony. As described previously by Kilaru et al. (2006), the *A. bisporus gpdII* promoter performed best with respect to the number of transformants with halos obtained and the distribution of size of halos within this group of positive transformants. Also as before, the experiment with the *S. commune Sc3* promoter showed by numbers of transformants with halos and distribution of halos, that the *Sc3* promoter is less active in monokaryon FA2222 than the *A. bisporus gdpII* promoter (Kilaru et al. 2006; Table 3). New in this study is the result on the *S. commune Sc4* promoter that performed comparably poorly in monokaryon FA2222 (Table 3).

In case of the transformations of monokaryon 218, the efficiency of the different promoters tested by laccase activity in transformant clones were not easy to determine due to the endogenous laccase production by the strain. However, from the overall picture, the tendency that the *gdpII* promoter performs best followed by the *Sc3* and *Sc4* promoter is also seen in the bulk analysis of 218 transformants (Table 4). To better test the effects of introduced promoter–laccase gene constructs, a subselection of transformants were cultivated for 6 days at 37°C in the liquid medium. Clones from the pYSK7 transformation generally showed much more laccase activity than clones of the other groups of transformations (Fig. 4). Second best were clones from the pYSK17 transformation, and third best were clones from the pYSK39 transformation. Again, the results suggest a hierarchy of promoter activities *gdpII* > *Sc3*> *Sc4*.

Table 3 Laccase activities of picked transformants of FA2222 monokaryon after two days of growth at room temperature on the solid minimal medium with 0.5 mM of ABTS

Plasmid (s)	**Promoter**	**Total clones tested**	**Transformant category**			
			-	+	++	+++
pCc100	-	48 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
pCc1001 + pYSK7	*gpdII*	41 (100%)	20 (50%)	9 (22.5%)	6 (15%)	5 (12.5%)
pCc1001 + pYSK17	*Sc3*	72 (100%)	41 (57%)	19 (26%)	9 (13%)	3 (4%)
pCc1001 + pYSK39	*Sc4*	71 (100%)	52 (73%)	15 (21%)	2 (3%)	2 (3%)

Table 4 Laccase activities of picked transformants of 218 monokaryon after two days of growth at room temperature on the solid minimal medium with 0.5 mM of ABTS

Plasmid (s)	**Promoter**	**Total clones tested**	**Transformant category**			
			-	+	++	+++
pCc100	-	48 (100%)	6 (13%)	25 (52%)	14 (35%)	0 (0%)
pCc1001 + pYSK7	*gpdII*	40 (100%)	0 (0%)	11 (28%)	19 (48%)	10 (26%)
pCc1001 + pYSK17	*Sc3*	57 (100%)	10 (18%)	27 (47%)	17 (30%)	3 (5%)
pCc1001 + pYSK39	*Sc4*	49 (100%)	5 (10%)	30 (61%)	8 (16%)	6 (12%)

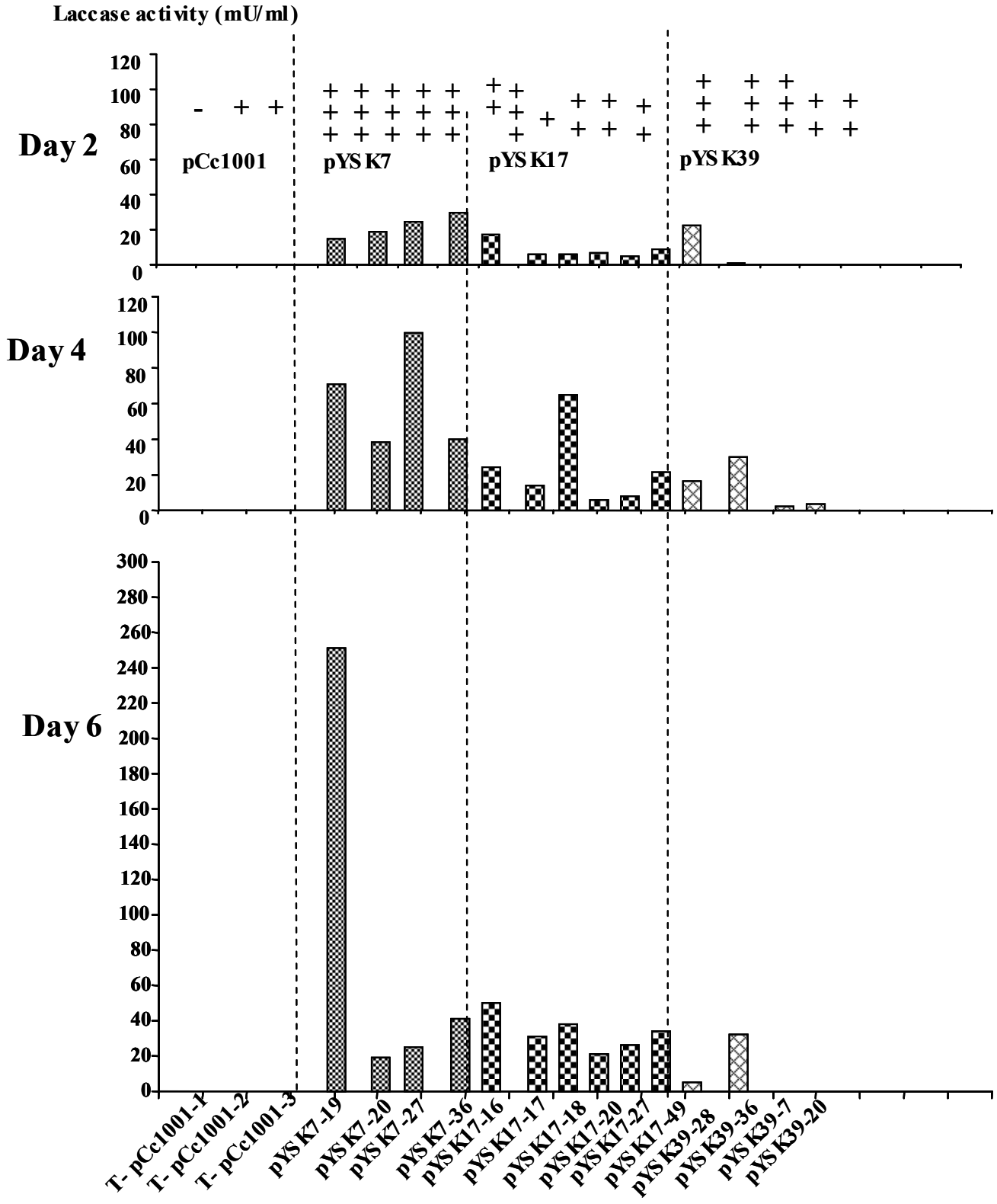


Figure 4. Laccase activities on YMG culture supernatants of selected 218 transformants. Supernatants of all clones (one culture per clone) were tested with ABTS-test at day 2, day 4 and day 6 of growth at 37°C, and measured the enzyme activities for the initiation clones are shown in the figure. Names of individual transformants are given at the bottom of the figure together with the activity category (-, +, ++, +++) they were grouped to in the previous plate test. From this experiment clones 19 and 20 of pYSK7, 16 and 17 of pYSK17, and 28 and 36 of pYSK39, clone 1, 2, and 3 of control were further selected for dikaryon formation with FA2222 transformants.

Highly laccase active FA2222 transformants (all categorized +++ in the solid medium test) and highly laccase active 218 transformants (see Fig. 4) were selected for dikaryon production. Dikaryons were produced between transformants of the same type of promoter (*gdpII*/218 X *gdpII*/FA22229, *Sc3*/218 X *Sc3*/FA2222, *Sc4*/218 X *Sc4*/FA2222), and of mixed combinations (*gdpII*/218 X *Sc3*/FA2222, *gdpII*/218 X *Sc4*/FA2222, *Sc3*/218 X *gdpII*/FA2222, *Sc3*/218 X *Sc4*/FA2222, *Sc4*/218 X *gdpII*/FA2222, *Sc4*/218 X *Sc3*/FA2222. In addition, a control dikaryon between pCc1001 transformants of the two monokaryons were constructed and dikaryons with one pCc1001 transformant and one transformant of *lcc1* (pCc1001/218 X *Sc3*/FA2222, pCc1001/218 X *Sc4*/FA2222).

All obtained dikaryons were grown in the same liquid medium (YMG). The laccase gene *lcc1* was used as genetic marker to observe the functions of the heterologous promoters, i.e., *gpdII, Sc3*, and *Sc4* in the *C. cinerea* dikaryons. In a first trial, precultures grown for 5 days on the solid YMG medium were used to inoculate liquid main cultures for laccase testing (Fig. 5). With the control dikaryon, no laccase activity was seen unlike in all other dikaryons, suggesting that in principle all foreign promoters are active in the dikaryon stage of *C. cinerea*. The best performances were seen with dikaryons *Sc3*/218 X *SC3*/FA22229, *gpdII*/218 X *gpdII*/FA2222, *Sc3*/218 X *Sc4*/FA2222) whereas combination *Sc4*/218 X *Sc4*/FA2222 and other mixed combinations (*gdpII*/218 X *Sc3*/FA2222, *gdpII*/218 X *Sc4*/FA2222, *Sc4*/218 X *gdpII*/FA2222, *Sc4*/218 X *Sc3*/FA2222, *gdpII*/218 X pCc1001 FA2222, *Sc3*/218 X pCc1001/FA2222, *Sc4*/218 X *Sc4*/ pCc1001) were less active. In this study, pure YMG medium was used with limited amounts of copper which is required for enzyme action. Kilaru et al. (2006) showed before that addition of low extra amounts of copper can lead in this medium with FA2222 transformants to much higher activities, likely due to stabilisation of the laccase proteins expressed in overexcess and providing them with the four copper atoms required per molecule for activity. In conclusion, the experiment presented in Fig. 5 does not yet allow to deduce a final definition of order of promoter activities in the dikaryon. Furthermore, since transformants of the same construct tend to differ in their laccase activities (Kilaru et al. 2006, Fig. 4 this work), more dikaryons of transformants from a same construct need to be tested.

In a repeat of the experiment (Fig. 6), liquid culture YMG medium with 0.1 mM of $CuSO_4$ was added. Best performing dikaryons in this experiment were pCc1001/281 X *gpdII*/FA2222, *gpdII*/218 X *gpdII*/FA2222, *Sc3*/218 X *Sc3*/FA2222. In conclusion, two of the former well performing transformants from the experiment shown in Fig. 5 were also the best in the experiment shown in Fig. 6. Still, as already indicated above more thorough analyses of these and further transformants will have to be done in the future.

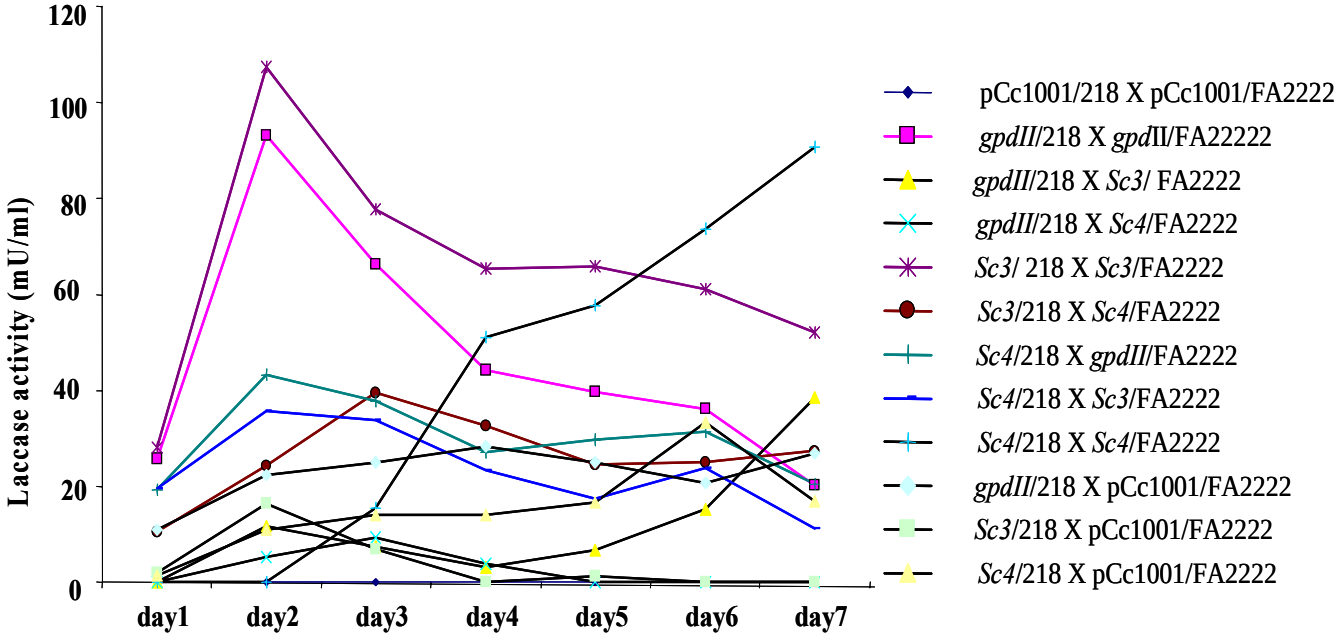


Figure 5. Laccase activity in YMG culture supernatants of various *C. cinerea* dikaryons that were grown at 37 °C in shaking culture. Dikaryons were grown in precultures for 5 days at 37°C on solid YMG agar. These cultures were homogenized and transformed into liquid YMG medium. [Note that the precultures of dikaryons *gdpII*/218 X pCc1001 FA2222, *Sc3*/218 X pCc1001/FA2222, *Sc4*/218 X pCc1001/218, and *Sc3*/218 X *gdpII* FA2222 were lost due to the bacterial infections].

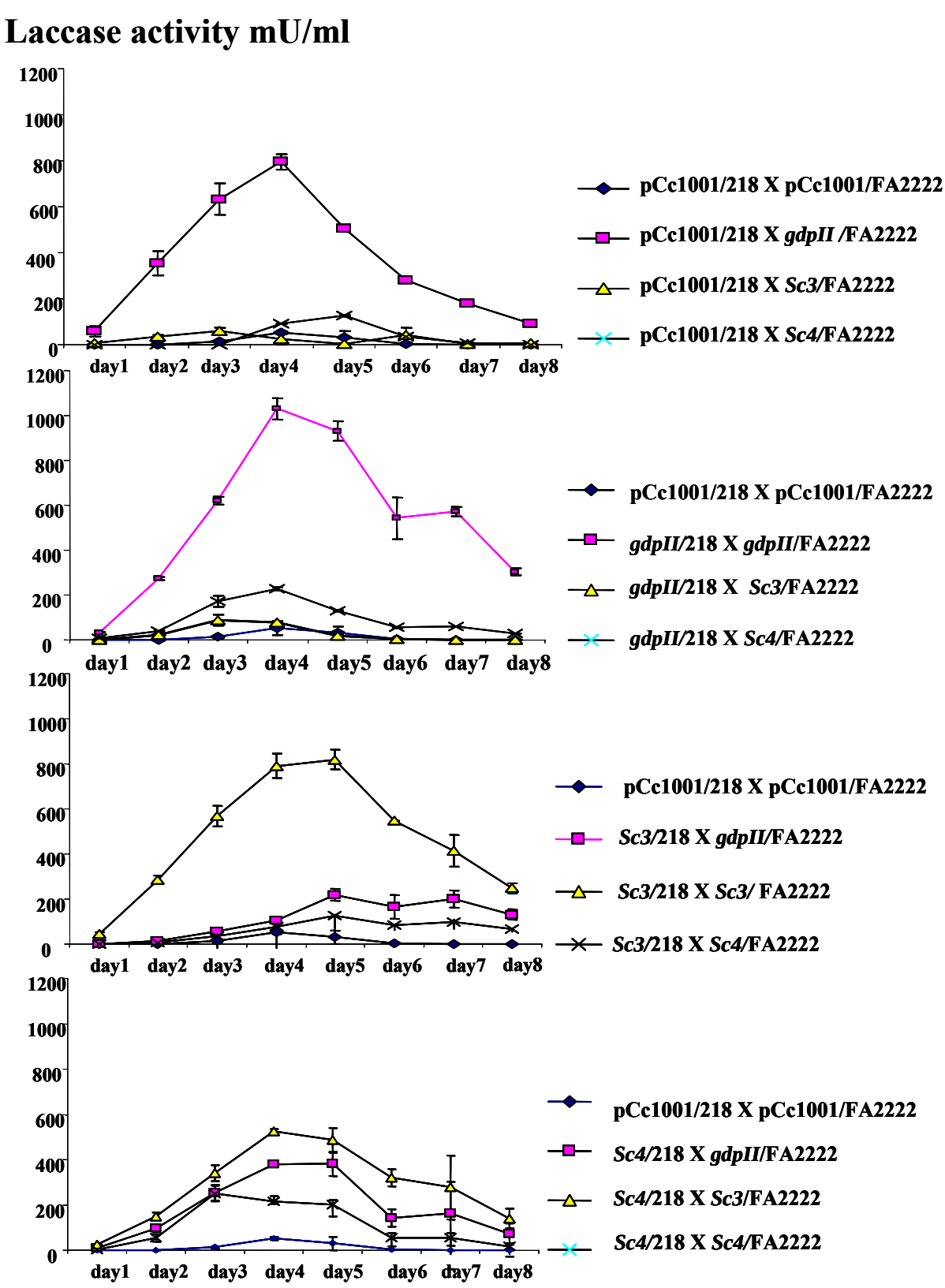


Figure 6. Laccase activity in YMG culture supernatants of various *C. cinerea* dikaryons that were grown at 37 °C in shaken culture. Dikaryons were grown in precultures for 5 days at 37 °C on solid YMG agar. These cultures were homogenized and transformed into liquid YMG medium with 0.1 mM of $CuSO_4$. Three replicates per dikaryon were analyzed and average values and standard deviations calculated.

9.4.3. Expression of laccase in the transformed dikaryons during fruiting body development

Day 3 primordia (size 0.2-0.3 cm), day 4 primordia (size 0.4-0-6 cm), immature fruiting bodies and mature fruiting bodies were harvested from dikaryons of transformants of strains 218 and FA2222 (Fig. 7). In case of 3 day primordia, 5 structures per dikaryon were combined and then grinded. In case of day 5 primordia, only one structure per dikaryon was analyzed. Of each one immature and mature fruiting body per dikaryon, cap and stipe were separated and tissues were grinded. Each of the tissue samples was tested semi-quantitatively for laccase activity by using ABTS as the substrate (weights of tissue were not determined before the enzyme test). Tissues reacted differentially in the laccase test. Depending on the enzyme activity, the color of the ABTS solution was changed to green-blue or dark-blue in the tissues samples which had accumulated laccase (Fig. 8). Samples which showed a dark blue color (Fig 8 **A**) were defined as "high laccase activity", sample with a blue-green color (Fig. 8 **B**) as "moderate laccase activity" and samples with a light blue color (Fig. 8 **C**) as "low laccase activity". However, there were differences in time needed to obtain the different degrees of color. Therefore, in Fig 9, compiling the results of samples of all tested dikaryons, next to the color code, times needed to obtain the color are also given.

Samples came from dikaryons *gdpII*/218 X *gdpII*/FA22229, *Sc3/*218 X *Sc3*/FA2222, *Sc4*/218 X *Sc4/*FA2222, *gdpII*/218 X *Sc3*/FA2222, *gdpII*/218 X *Sc4*/FA2222, *Sc3*/218 X *gdpII*/FA2222, *Sc3/*218 X *Sc3*/FA2222, *Sc3*/218 X *Sc4/*FA2222, *Sc4/*218 X *gdpII/*FA2222, *Sc4*/218 X *Sc3/*FA2222, *Sc4*/218 X *Sc4/*FA2222, pCc1001/218 X *Sc3*/FA2222, pCc1001/218 X *Sc4*/FA2222 and from a dikaryotic control pCc1001/218 X pCc1001/FA2222.

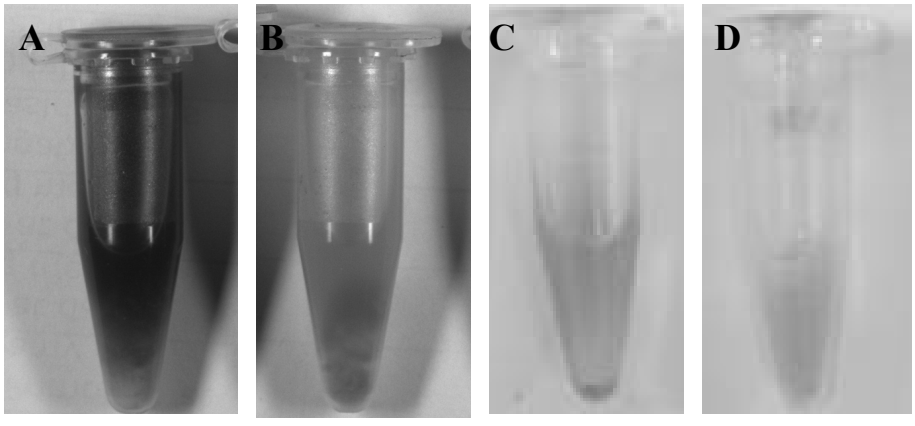


Figure 7. Stages of fruiting body development of *C. cinerea* used in analysis of laccase expression (courtesy of M. Navarro-González). **(A)** Day 3 primordium (size 2-3 mm). (**B)** Day 5 primordium (size 4-6 mm). The day refers to the time primordia took to develop from the point of initiation of fruiting body development (Kües 2000) to the stage at the day of harvest. Primordia were harvested at the beginning of the day period in the 12 hours light/day and 12 hours dark/day cycle. The fungi were kept in the standard conditions for fruiting. **(C)** Immature fruiting bodies were harvested at the end of the light period at day 5 of fruiting body development. **(D)** Mature fruiting bodies at the beginning of the light period of day 6.

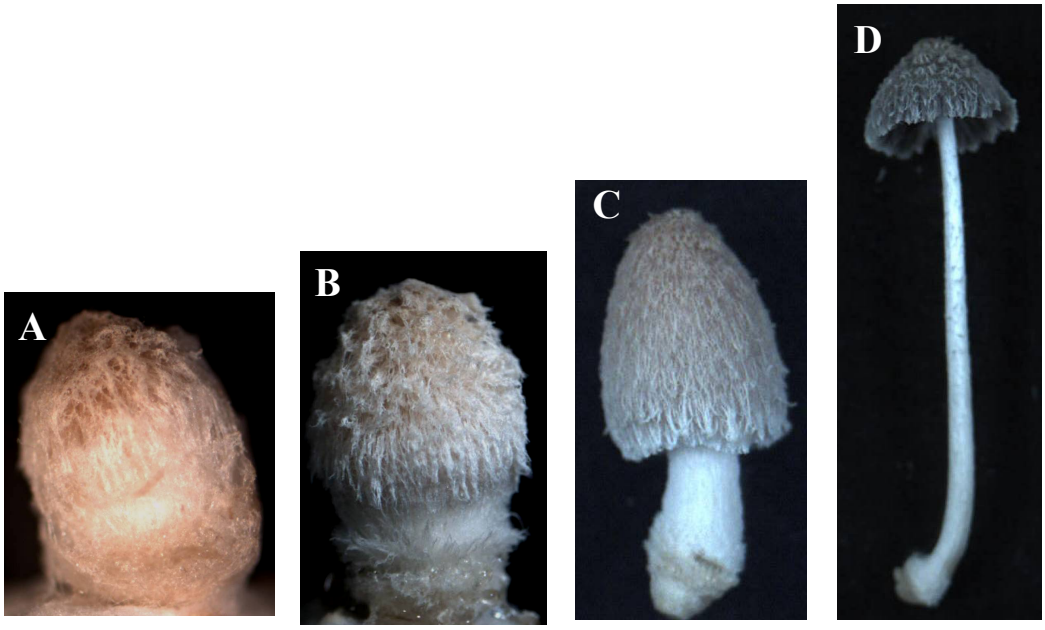


Figure 8. Scale was assigned to the defined level of laccase activity which occurred by oxidization of ABTS in tissue samples of *C. cinerea* fruiting structures; High laccase activity (**A**), moderate laccase activity (**B**), low laccase activity (**C**), and control buffer with ABTS (light green color) (**D**). Sample **A** shows tissues of a mature fruiting body cap, sample **B** of a immature fruiting body. Sample **C** shows the sample of day 4 tissues.

	pCc1001218 x gpd FA2222	pCc1001218 x Sc3 FA2222	pCc1001218 x Sc3 FA2222	pCc1001218 x pCc1001FA2222	gpdII218 x gdpIIFA2222	gpdII218 x Sc 3 FA2222	gpdII218 x Sc4 FA2222	gpdII218 x pCc1001FA2222	Sc3218 x gpdIIFA2222	Sc3218 x Sc3FA2222	Sc3218 x Sc4FA2222	Sc3218 x gpCc1001FA2222	Sc4218 x gpdIIFA2222	Sc4218 x Sc3FA2222	Sc4218 x Sc4FA2222	Sc4218 xpCc1001FA2222
Day3 primordia (size 2-3 mm)	X	20 min	On	No	No	X	1 h	No	1 h	No	On	No	On	On	No	On
Day5 primordia (size 4-6 mm)	X	20 min	No	No	On	On	On	On	20min	X	2min	X	On	On	X	X
Immature FB (Stipe)	On	On	On	On	On	On	On	On	5 min	15min	On	20min	1.3 h	15 min	On	On
Immature FB (Cap)	On	On	On	On	1mim	On	On	On	5 min	3 min	On	2h	On	15 min	On	On
Mature FB (Stipe)	On	20 min	20 min	On	On	On	On	2 h	2 min	20 min	On	20 min	On	On	45 min	On
Mature FB (Cap)	On	20 min	20 min	On	On	On	On	45min	2 min	20min	On	On	On	On	30 min	On

Figure 9. Profile of laccase activities in primordia and fruiting body (FB) tissues of dikaryons carrying laccase gene *lcc1* under control of heterologous promoters (*gdpII, Sc3, Sc4*) and pCc1001. The control is the dikaryon transformed only with plasmid pCc1001 which carrying a wild type *trp1*$^{+}$ Gene. **No**: no laccase activity occurred, **On**: incubated over night, **min** and **h**: the time after which a laccase reaction had been observed.

In the control dikaryon without extra laccase gene (pCc1001/218 X pCc1001/FA2222), in the primordia sample, no laccase activity was observed. Only low laccase activity was observed in the cap of the immature and mature fruiting body, and high activity in the mature fruiting body at harvesting of the mature fruiting body (Fig. 9) where the degeneration of the structure starts by autolysis (Kües 2000). The natural laccase activity may therefore relate to the sequence of processes in fruiting body development, particularly spore production and coloring and tissue degeneration for the release of the matured spores.

In most cases of dikaryons transformed with the laccase genes *lcc1* under control of a foreign promoter, laccase expression was also observed in the early stages of fruiting body development and that usually with much higher activity (Fig. 9). From the various results shown in Fig. 9, one can therefore suspect that all three foreign promoters are active during fruiting body development of *C. cinerea*. The *Sc4* promoter of *S. commune* is a promoter specially expressed during fruiting in its native host (Schuren and Wessels 1990; Wessels et al. 1991a). Compared to the expression of laccase in the vegetative mycelia of the transformed dikaryons (Fig. 6), it appears that there is a tendency of much higher laccase activity during fruiting from the *Sc4* promoter (Fig. 9). Similarly, as a tendency the *Sc3* promoter appears to be more active during fruiting in *C. cinerea* than the constitutive *gdpII* promoter (Fig. 9). Also the *Sc3* promoter in its native host *S. commune* showed some activity in the fruiting body (Van Wetter et al. 2000). On the whole, however, one has to take care not to over-interpret the data from this experiment, since only one sample from one dikaryon per transformation case was tested and that only semi-qualitatively. Many more dikaryons have to be analyzed before a more reliable conclusion on the behavior of the heterologous promoters during fruiting in *C. cinerea* is allowed.

9.5. Conclusion

This chapter presents a number of interesting observations from transformations of DNAs of heterologous basidiomycetes into *C. cinerea*. In terms of homeodomian transcription factors encoding mating type genes, evidence is presented that both types of mating type genes *HD1* and *HD2* from *U. maydis* as well as from *S. commune* can affect clamp cell development in *C. cinerea*. However, such reactions were only transiently observed only in sectors of the colonies. It is possible that the foreign mating type proteins react only poorly with resident mating type proteons in order to switch on clamp cell production and that this postulated poor protein-protein interaction is quickly dissolved already upon slight changes in the environment.

Regarding the activity of heterologous promoters in *C. cinerea*, one can deduce from the presented data that all three tested promoters, the developmentally regulated *Sc3* and *Sc4* promoters from *S. commune* and the constitutive *gdpII* promoter from *A. bisporus* are active in monokaryons,

dikaryons and fruiting bodies of *C. cinerea*. It appears that the *gdpII* promoter is best in the vegetative mycelium of monokaryons and dikaryons and that the *Sc4* promoter is the best in the fruiting bodies, followed by the *Sc3* promoter.

Further research has to confirm the observations on foreign promoters presented in this chapter and also to solve the question, why expression of clamp cell in transformants of mating type genes from *U. maydis* and *S. commune* is only transient.

9.6. References

Alves, A. M. C. R., E. Record, A. Lomascolo, K. Scholtmeijer, M. Asther *et al.*, 2004 Highly efficient production of laccase by the basidiomycetes *Pycnoporus cinnabarinus*. Appl. Environ. Microbiol. **70:** 6379-6384.

Bardwell, L., J. G. Cook, C. J. Lnouye, and J. Thorner, 1994 Signal propagation and regulation in the mating pheromone response pathway of the yeast *Saccharomyces cerevisiae*. Dev. Biol. **166:** 363-379.

Binninger, D. M., C. Skrzynia, P. J. Pukkila, and L. A. Casselton, 1987 DNA-mediated transformation of the basidiomycete *Coprinus cinereus*. EMBO J. **6:** 835-840.

Bölker, M., Urban M., and Kahmann, R. 1992. The *a* mating type locus of *Ustilago maydis* specifies cell signaling components. Cell **68:** 441-450.

Dwivedi, R. C., 2006 *Cell Wall-Associated Redox Enzymes in White Rot Fungi*. Ph.D. Thesis, Georg-August-University of Göttingen, Göttingen, Germany.

Granado, J. D., K. Kertesz-Chaloupková, M. Aebi, and U. Kües, 1997 Restriction enzyme-mediated DNA integration in *Coprinus cinereus*. Mol. Gen. Genet. **256:** 28-36.

Gregory, B. C., M. Kirby, M. K. Cheung, M. Riquelme, T. J. Elliott *et al.*, 2005 Efficient GFP expression in the mushrooms *Agaricus bisporus* and *Coprinus cinereus* requires introns. Fungal Genet. Biol. **42:** 191-199.

Hull, C. M., R. C. Davidson, and J. Heitman, 2002 Cell identity and sexual development in *Crytococcus neoformasns* are controlled by the mating- type- specific homeodomain protein Sxilα. Genes Dev. **16:** 3046-3060.

Herskowitz, I., J. Rine, and J. Strathen, 1992 Mating type determination and mating-type interconversion in *Saccharomyces cerevisiae,* pp. 583-65 in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression*, edited by E. W. Jones, J. R. Pringel and J. R. Broach, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

James, T. Y., P. Srivilai, U. Kües, and R. Vilgalys, 2006 Evolution of the bipolar mating system of the mushroom *Coprinellus disseminatus* from its tetrapolar ancestors involves loss of mating-type-specific pheromone receptor function. Genetics **172:** 1877-1891.

Kilaru, S., P. J. Hoegger, A. Majcherczyk, C. Burns, K. Shishido *et al.*, 2006 Expression of laccase gene *lcc1* in *Coprinopsis cinerea* under control of various basidiomycetous promoters. Appl. Microbiol. Biotechnol. **70:** 200-210.

Kothe, E., 2001 Mating-type genes for basidiomycete strain improvement in mushroom farming. Appl. Microbiol. Biotechnol. **56**: 602-612.

Kronstad, J. W., 1997 Mating type in filamentous fungi. Annu. Rev. Genet. **31:** 245-276.

Kronstad, J. W., and S. A. Leong, 1990 The *b* mating-type locus of *Ustilago maydis* contains variable and constant regions. Genes Dev. **4:** 1384-1395.

Kronstad, J. W., and C. Staben, 1997 Mating type in filamentous fungi. Annu. Rev. Genet. **31:** 245-276.

Kües, U., 2000 Life history and developmental processes in the basidiomycete *Coprinus cinereus*. Microbiol. Mol. Biol. Rev. **64:** 316-353.

Kües, U., T. Y. James, R. Vilgalys, and M. P. Challen, 2001 The chromosomal region containing *pab-1*, *mip*, and the *A* mating type locus of the secondary homothallic homobasidiomycete *Coprinus bilanatus*. Curr. Genet. **39:** 16-24.

Messerschmidt, A., 1997 *Multi-Copper Oxidases*. World Scientific, Singapore.

Mulder, G. H., and J. G. H. Wessels, 1986 Molecular cloning of RNAs differentially expressed in monokaryons and dikaryons of *Schizophyllum commune*. Exp. Mycol. **10:** 214-227.

Muraguchi, H., Y. Ito, T. Kamada, and S. O. Yanagi, 2003 A linkage map of the basidiomycete *Coprinus cinereus* based on random amplified polymorphic DNAs and restriction fragment length polymorphisms. Fungal Genet. Biol. **40:** 93-102.

Raper, J. R., 1966 *Genetics of Sexuality in Higher Fungi*. Ronald Press, New York.

Robertson, C., K. A. Bartholomew, C. P. Novotny, and R. C. Ullrich, 1996 Deletion of the *Schizophyllum commune* Aα locus: the roles of the Aα *Y* and *Z* mating–type genes. Genetics **144:** 1437-1444.

Van Wetter, M. A., H. A. B. Wösten, and J. G. H. Wessels, 2000 SC3 and SC4 hydrophobins have distinct roles in formation of aerial structures in dikaryons of *Schizophyllum commune*. Mol. Microbiol. **36:** 201-210.

Velagapudi, R., 2006 *Extracellular Matrix Protein in Growth and Fruiting Body Development of Straw and Wood Degrading Basidiomycetes*. Ph.D. Thesis, Georg-August-University of Göttingen, Göttingen, Germany.

Wessels, J. G. H., G. H. Mulder, and J. Springer, 1987 Expression of dikaryon–specific and non-specific mRNA's of *Schizophylum commune* in relation to environmental conditions and fruiting. J. Gen. Microbiol. **133:** 2557-2561.

Wösten, H. A. B., 2001 Hydrophobins: multipurpose proteins. Annu. Rev. Microbiol. **55:** 625-645.

CHAPTER 10

General Discussion

10.1. Organisms of study of this thesis

Based on sexual reproduction, basidiomycetes can be divided into two groups i.e., homothallic and heterothallic species. In the heterothallic species, i.e. for example, *Coprinopsis cinerea*, *Schizophyllum commune, Ustilago maydis* and *Coprinellus disseminatus*, sexual reproduction is regulated by the mating type genes. *C. cinerea* is the main study organism of this thesis (see below). The other three species provided genes, respectively promoters that were analyzed in this work upon transformation in the heterologous host *C. cinerea*.

The dung fungus *C. cinerea* (formerly called *Coprinus cinereus*, Redhead et al. 2001) is an excellent model to study fruiting body development in the basidiomycetes. This saprophyte easily grows in the laboratory and completes its life cycle within two weeks allowing access by classical genetics (Kües 2000; Walser et al. 2001). More than that, it is possible to transform the fungus (Granado et al. 1997) and the genome of the fungus had been released by the Broad Institute (see http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/).

The life cycle of the heterothallic *C. cinerea* begins with the germination of basidiospores into monokaryons that contain one type of haploid nuclei. For sexual reproduction, each monokaryon has to mate with another compatible monokaryon of different mating type to form a fertile dikaryon. The dikaryon differs from the monokaryon by formation of clamp cells at the hyphal septa and it has two haploid nuclei in its cells that differ in their mating type genes. On the dikaryon, under defined environmental conditions, fruiting bodies develop in which in the specialized basidia, in which karyogamy and meiosis occur with finally results in the formation of four basidiospores per basidium. Each basidiospore obtains one haploid nucleus from meiosis, and there is a post-meiotic nuclear division in the spores. As a result, each unicellular basidiospore contains two identical haploid nuclei (Kües 2000; Kües et al. 2004). As a subsidiary cycle, on both the monokaryon and the dikaryon, *C. cinerea* can form the haploid unicellular asexual spores known as oidia (Kües et al. 2002).

S. commune and *C. disseminatus* are both higher basidiomycetes that are able to attack wood of broad-leaf trees. *S. commune* in addition can attack weakened living trees (Runge 1975; Griesser 1992; Wessels 1993; Dix and Webster 1995; Peddireddi et al. 2005). *S. commune* is like *C. cinerea* a typical model species for studying general development of higher basidiomycetes (also known as homobasidiomycetes). The main life cycle of *S. commune* follows that of *C. cinerea* (Raper 1966), but the fruiting bodies differ in shape (fan–shape in case of *S. commune*, a typical mushroom shape with stipe and cap in case of *C. cinerea* (Wösten and Wessels 2006; Kües et al. 2004). Unlike *C. cinerea*, *S. commune* does not form oidia on its monokaryotic or dikaryotic mycelia. *S. commune* and *C. cinerea* are both tetrapolar, i.e., sexual development in these species is controlled by two

different mating type loci (Kües 2000; Kothe 2001). The mating type genes of *S. commune* have been cloned in the past and their functions in its original host were studied (Giasson et al. 1989). The genes at this loci are homologous between the two species and an obvious question to address was whether they function not only in the own host but also in a foreign species (Kües and Casselton 1993; Casselton and Olesnicky 1998). As developmentally regulated genes, *SC3* and *SC4* encoding hydrophobin proteins were also available from this fungus (Wessels et al. 1995). The *A* mating type genes of *S. commune* and the promoters of the *SC3* and *SC4* genes of *S. commune* were transformed in this work into *C. cinerea.*

In contrast to *C. cinerea* and *S. commune*, *C. disseminatus*, having also a typical homobasidiomycete life cycle and fruiting bodies in appearance as *C. cinerea*, is a bipolar species. Bipolarity was the reason to clone and study the mating type genes of this species (James et al. 2006). Since there is no transformation system available for *C. disseminatus*, functionality of cloned mating type genes was tested in *C. cinerea.*

U. maydis is a plant pathogenic fungus. However, this heterobasidiomycete does not attack woody plants but it is specialized on maize (Holliday 1961). *U. maydis* is seen as the model fungus for heterobasidiomycete plant pathogens (Day et al. 1971; Banuett and Herskowitz 1989; Grandel et al. 2000). Also *U. maydis* is tetrapolar with two mating type loci that are homologous to the genes at the two mating type loci in the higher basidiomycetes (Rowell 1955; Kües and Casselton 1993; Casselton and Olesnicky 1998; Urban et al. 1996). However, since *U. maydis* is a heterobasidiomycete, it does not form mushrooms (Banuett and Herskowitz 1989). Instead, in planta at the end of a growth period, diploid unicellular teliospores form of which in spring time the pro-basidia germinate. From the pro-basidium, basidiospores known as sporidia bud off (Grandel et al. 2000). From this species, mating type genes were also available for studying in *C. cinerea* in order to follow how conserved essential elements in control of sexual development in basidiomycetes are. By historical reasons, the mating type genes of *U. maydis* being homologous to the *A* mating type genes of the higher basidiomycetes are known as *b* (Casselton and Olesnicky 1998).

10.2. Mutation in fruiting in *C. cinerea*

Basically, *C. cinerea* develops from the vegetative dikaryotic mycelium to the mature fruiting body within 1 week under appropriate environmental conditions. However, some dikaryons stop development already at initiation of fruiting body formation, i.e. at the stage of mycelial aggregation, or within primordia formation due to defects in various gene functions. From work of other authors it was known that in nature defects of various genes occur within the natural

populations of *C. cinerea* strains that do not or only suboptimal function in fruiting body development (Moore 1981, 1998).

There are three possibilities of how defect genes in nature may occur in a *C. cinerea* dikaryon: i) homozygous dominant, ii) heterozygous dominant and iii) homozygous recessive. While in the homozygous dominant and in a homozygous recessive situation, a mutant phenotype will be established, in the heterozygous dominant situation, only dominant mutated genes and not recessive mutated genes will manifest themselves in the phenotype. For the interested reader, it remains to be mentioned that although the two haploid nuclei from the parental monokaryons do not fuse in the dikaryon, the principle genetic situation of a dikaryotic cell is comparable to that of a diploid cell: there are two sets of chromosomes within one cell whose genes can be complemented to each other - hence the words homozygous and heterozygous. Within the *C. cinerea* dikaryons, recessive genes are only expressed in the phenotypes when present in the homozygous state. According to several evidences that were given by Takemura and Kamada (1969, 1970, 1972) and by Moore (1969, 1970, 1972, 1981), there are many unknown recessive genes present in nature that are silent in heterozygous dikaryons of *C. cinerea* and these reside within the natural gene pool of the species. In contrast, dominant mutations are rarely found in nature (Muraguchi and Kamada 2000). Selection for sexual reproduction disfavors their maintenance in the gene pool, unless it is a mutation that affects just the shape of the mushroom (Muraguchi and Kamada 2000) or it is a mutation that allows fruiting already on the monokaryon. The *fis*^{c} mutation isolated in the year 1971 for example is a such a mutation of the later case (Uno and Ishikawa 1971), and also the *Cop5D* mutation isolated in the year 1998 (Murata et al. 1998; Muraguchi et al. 1999; Muraguchi and Kamada 2000). Sequencing demonstrated that in the two mutants the same gene was affected, *pcc1* encoding a potential transcription factor (Murata et al. 1998). Such monokaryotic fruiting mutants obviously occur in certain frequencies within nature. They nevertheless have disadvantages compared to the dikaryons, for example in the frequencies of initiation of fruiting body development and in the correct formation of mushrooms with well structured caps and stipes (Bottoli and Kües, personal communication). This is likely also due to suboptimal genes in later stages of fruiting. Moreover, fruiting on the monokaryon avoids mixing of the *C. cinerea* gene pool which is also seen as a reason as why in nature fruiting dikaryotic strains are found in a nearly exclusive majority.

To study the functions of genes in fruiting body development of *C. cinerea*, Takemaru and Kamada used macerated mycelium of *C. cinerea* dikaryons and performed UV mutagenesis and/or chemical mutagenesis with NG (*N*-methly-*N′*-nitro-*N*-nitroso-guanidine). Surprisingly, they found abnormalities in fruiting body development in frequencies of over 10% of the tested clones. A total of 1,594 developmental variants were identified amongst 10,641 tested isolates (Takemaru and

Kamada 1969, 1970, 1972). Takemaru and Kamada (1972) suggested three causes for the high amount of variants observed in their studies as listed in the following: i) influence of factors other than genes, ii) mutations in dominant genes and iii) easy access of fruiting genes in mutagenesis. Moore (1981) deduced subsequently from genetic crosses of natural monokaryotic strains that there are many recessive genetic defects in fruiting body development present within the natural gene pool of *C. cinerea*. Studies in our group for example showed that a mutation by transposon insertion occurred in a gene necessary for the stage of mycelial aggregation at the beginning of the fruiting process of *C. cinerea* (Clergeot et al. unpublished) in the monokaryotic strain that was chosen for establishing the *C. cinerea* genome (Kües, personal communication).

Quite unintended, the genome sequencing project has thus uncovered a new mutation in fruiting in *C. cinerea*. Moreover, sometime spontaneous mutations occur in crosses of *C. cinerea.* UFO1 found in crosses in this work (chapter 2) is such a spontaneous mutant which is characterized by a short stipe (Srivilai et al. 2005). Interesting, mutant UFO1 also carries a defect in a basidiospore formation gene (*bad* or *spo*) that came from the parental monokaryon 218 (Pukkila 1993). Strain UFO1 is thus an example that a single strain can accumulate several different natural mutations in the fruiting pathway.

Till now, very few genes of fruiting body development of *C. cinerea* have been studied by gene cloning for analysing the respective functions. As a first gene, the pileus-specific *ich1* (*ichijiku1*) gene was cloned from an *ich1* mutant that fails to differentiate the pileus tissue at the apex of the primordial stipe (Muraguchi and Kamada 1998). The *eln2* (elongationless 2) gene is a constitutively expressed gene that encodes a novel type of a microsomal cytochrome P450 enzyme, termed CYP502. A dominant *eln2* mutation affects stipe tissue formation in the primordia and the defect results in dumpy fruiting bodies with short stipe (Muraguchi and Kamada 2000). By transformation with a plasmid carrying a wild type *eln3* gene, Arima and co-workers could rescue the wild type phenotype in the *eln3-1* mutant which prior to transformation was characterized by a defect in the stipe elongation (Arima et al. 2004). A mutation in the *cfs1* encoding a potential cyclopropane fatty acid synthase gene affects the transition of the primary hyphal knots to the secondary hyphal knots. Also this gene was found by mutant complementation (Liu 2001; chapter 4 of this thesis). Most interesting mutants in *C. cinerea* are those that are defective in their mating type genes, with the consequence that fruiting body development occurs without mating to another strain (Swamy et al. 1984).

The *C. cinerea* homokaryon AmutBmut is such a strain that contains mutations in both the *A* and the *B* mating type loci. Consequently, the strain forms fruiting bodies without mating with another compatible strain. In addition, it forms clamp cells at the hyphal septa that in certain instances fuse to the subapical cells. Strain AmutBmut is therefore not referred to as monokaryon

but it is called dikaryon-like homokaryon (Swamy et al. 1984). Having this homokaryon AmutBmut allowed to find and understood more recessive mutant genes in *C. cinerea*. Mutants of the self-compatible homokaryon AmutBmut are easy to generate by using either the classical mutation methods such as UV-treatment or by modern REMI (Restriction Enzyme-Mediated Integration) mutagenesis *via* DNA transformation. Large collections of mutants homokaryon Amut Bmut were produced (Granado et al. 1997; Kües et al. unpublished) that can be used for studying individual steps in fruiting body development. In the past, genetic analysis of these mutants turned out to be difficult to perform due to the lack of co-isogenic compatible monokaryons (Liu 2001; Liu et al. 1999). As an example, the mutant 6-031, obtained from homokaryon AmutBmut by UV-mutageneis, was suspected to contain several mutated genes in the fruiting process induced by the UV-treatment. Crosses of the mutant to unrelated monokaryons suggested that there were a *skn* defect (secondary hyphal knot formation), affecting the developmental transition of the primary hyphal knots to the secondary hyphal knots and at least one *mat* (primordia maturation) mutation blocking the maturation of primordia into the fruiting bodies. However, since initiation of fruiting body development in the progenies was extremely low (in 20% of test homokaryotic clones being self-compatible due to inheritance of the two mutated mating type loci), definite conclusions on defects in mutant 6-031 could not be deduced from crosses performed by Liu (Liu 2001; Liu et al. 1999). These crosses performed by Liu however once more emphasized that within the natural gene pool of *C. cinerea* there are many recessive genes that negatively affect the fruiting process in the species.

In contrast to *C. cinerea*, in *S. commune* co-isogenic monokaryotic strains had been in use since 1960. In crosses between mutants of these co-isogenic strains, inheritance patterns are obtained within the progenies that are easily to analyse by the Mendelian´s rules (Raper 1996; Wessels 1997). It was thus the target of this thesis to generate monokaryotic strains of *C. cinerea* that are co-isogenic to homokaryon AmutBmut and that do not carry any genes negatively affecting fruiting.

10.3. Co-isogenic monokaryotic strains of *C. cinerea* homokaryon AmutBmut with compatible mating types

Co-isogenic strains are principally constructed by repetitive backcrosses with a fixed parental strain. According to the formula: $C(n) = 2c(1/2)^n = c(1/2)^{n-1}$ (Leslie 1980), the average number of chromosome tips remaining allogenic in repeated backcrosses with random chromosome distribution can be predicted and determined when C(n) is the average number of chromosome tips remaining allogenic, c is the haploid chromosome number (13 in *C. cinerea*; Pukkila and Lu 1985) and n is the number of the backcrosses performed.

We constructed co-isogenic monokaryotic strains of *C. cinerea* with different mating type specificities *A42B42* from monokaryon JV6 and *A3B1* from monokaryon 218 by repetitive backcrosses with a parental self–compatible homokaryon AmutBmut (chapter 3 of this thesis). Within the progenies, the mating type genes of *C. cinerea* were independently distributed according to the Mendelian genetic laws. Regarding the colony growth and fruiting body development, it was very clearly found that the F1 progenies had a high genetic variation much more than the F5 an F6 progenies. Following the law of Leslie (1980), within the F5 and F6 generations of cross homokaryon AmutBmut x monokaryon JV6, 96.6% and 98.3% of all genes, respectively, should originate from homokaryon AmutBmut. Within the F5 and F6 generations of cross homokaryon AmutBmut x monokaryon 218, 97% and 98% of all genes, respectively, should originate from homokaryon AmutBmut. In principle, this high level of co-isogenisation satisfies the criteria in Mendelian genetic analysis of crosses between the obtained monokaryons of different mating types and the mutants of the co-isogenic homokaryon AmutBmut being self–compatible due to the mutated *A* and *B* mating type genes (see below). To access the quality of co-isogenic strains, progenies of both series of crosses were then selected to perform a more thorough study on fruiting behavior. In the F6 generation, it was found that 95% of that part of the progeny formed mature fruiting bodies, whereas the other 5% did not (see chapter 3). This might be due to effects of environmental factors, but this also could be caused by a low level of mutations occurring during meiosis (see strain UFO1 as an example of such a spontaneous mutation occurring in meiosis, chapters 2 and 3 of this thesis).

Molecular markers known from the molecular mapping of two *C. cinerea* strains (Muraguchi et al. 2003) were used with our co-isogenic strains to confirm the quality of co-isogenisation. In additionally, primers of the *C. cinerea* spacer region of the hydrophobin genes *coh5-coh4* and of the laccase gene *lcc15* (Velagapudi 2006; Kilaru et al. 2006) were also used in assessment of the quality of co-isogenic strains. Particularly, the *coh5-coh4* spacer region and the *lcc15* gene were interesting to follow up since these genes are linked to the *B* and *A* mating type genes, respectively (Srivilai et al. unpublished). Our results showed that all chromosomes in the F6 generation but those with the mating type genes come from homokaryon AmutBmut. Genes from the mating type chromosomes came from the parental monokaryons JV6 and 218, respectively. This is not surprising, since selection in crosses were focussed on their mating type genes. Homologous recombination during meiosis in close vicinity to the mating type genes would be required between the chromosomes with the mating type genes from homokaryon AmutBmut and those of the monokaryons in order to obtain the AmutBmut non-mating type genes genes on the chromosomes linked to the foreign mating type genes. Interestingly, our results showed that an approximately tenfold higher recombination rate occurs between genes on chromosome X carrying the *B* mating

type locus and between genes on chromosome I carrying the *A* mating type locus (see chapter 3 of this thesis for more details). Due to the low recombination frequency in the chromosomes with the *A* mating type genes, within the F6 progeny of both crosses, we found the molecular markers which originated from the parental monokaryon JV6 (*pab1*$^{+}$, 1.5 kb *lcc15* fragment) and 218 (at least *pab1*$^{+}$, BA4-3100B=), respectively (see table 4 in chapter 3). On the chromosome with the *B* mating type genes in some but not all of the clones in the F6 generations, we however found the molecular markers G19-1700+, A10-1400+, E4-1400+ and 1.75 kb *coh5- coh4* spacer originating from the *B* chromosome of homokaryon AmutBmut (see table 4 in chapter 3 of this thesis). In conclusion, whilst recombination occurred between *B* mating type chromosomes, at least closer to the mating type genes, there is still a genetic variation between homokaryon AmutBmut and co-isogenic monokaryons obtained from monokaryon JV6 and 218 in the co-isogenic strain process by repetitive backcrosses to homokaryon Amut Bmut.

10.4. An essential gene for fruiting body initiation in the basidiomycete *C. cinerea*

The AmutBmut UV mutant 6-031 forms primary hyphal knots in the aerial mycelia, but fruiting body development is arrested at the transition state to the secondary hyphal knots due to the *skn1* (secondary hyphal knot formation gene 1), or *cfs1* (cyclopropane fatty acid synthase gene 1) gene defect, as the mutated gene was called after its function became obvious by cloning and sequencing (Liu 2001). The mutant *cfs1* allele was found to carry a T to G transversion, leading in the protein product to an amino acid substitution (Y441D) in a domain that is suggested to be involved in the catalytic function of the protein and/or in membrane interaction (Liu et al. 2006).

First complementation analysis of gene *cfs1* by Liu (2001) was done in the original mutant 6-031. However, whilst the defect of *cfs1* in fruiting initiation was overcome, mature fruiting bodies were never obtained from her study. Although, certain structure deformation of the primordia suggested that gene *cfs1* might also be required in later stages of primordia development. However, this was not fully clear as there were also evidences for mutations additionally to the *cfs1* gene to be present in the mutated strain 6-031. The complete wild type *cfs1* gene was isolated originally on a cosmid with a large fragment of genomic DNA from the wildtype homokaryon AmutBmut (Liu 2001). A normal primordia phenotype was achieved in both the transformation of mutant 6-031 performed by Liu (2001) as well as of strain OU3-1 preformed in this thesis, but only when gene *cfs1* was embedded at both sides in at least 4.0 kb of the native flanking DNA. Truncations of the flanking DNA lead in both strains to the reduction in transformation frequencies and to a failure in correct primordia structure formation, which emphasized that the *cfs1* gene is also needed at the later stages of development. With smaller DNA fragments containing only the *cfs1* gene, there was a block very early in primordia development (chapter 4 in this thesis and Liu 2001). A transcription

profile of gene *cfs1* during vegetative growth and fruiting performed by Liu (2001) supports this conclusion that there are additional functions of the gene in later stages of primordia development. In further transformations with internally deleted genes, we found that the *cfs1* neighboring genes *arf1*, *kin1* and *glt1* did not influence directly the initiation fruiting body development, but that the transcription profiles of *arf1* and *kin1* probably influence the expression of *cfs1* gene during early and later stages of fruiting body development. Transformation results with inactivated *art1, kin1* and *glt1* copies revealed that the functional expression of *cfs1* gene required the presence but not the functionality of the neighboring genes. Furthermore, the genetic data and the transformation data on strain OU3-1 showed that the earlier observed block in maturation of primordia in transformants of mutant 6-031 with the cosmid that contains the *cfs1* gene and other larger neighboring DNA fragments was caused by an yet unknown mutated gene *mat* (chapter 4 in this thesis and Liu 2001). In this study (chapter 3), a genetic cross of mutant 6-031 with the co-isogenic strain monokaryon PS001-1 and PS002-1 clearly demonstrated that mutant 6-031 carries next to gene *cfs1* an additional mutation (*mat*) that causes a block in primordia maturation and that there is also in mutant 6-031 another maturation (*bad*) that blocks basidiospore formation. Within the progenies of cross 6-031 and PS001-1 and of cross 6-031 and PS002-1, these mutations were separated from each other. Strain OU3-1 obtained from the cross PS001-1 X 6-031 has the genotype *A43mut, B43mut, pab1, cfs1, mat*$^{+}$*, bad*+. This strain was thus used to investigate the functions of *cfs1* gene in fruiting body development further to the results that were obtained by the study of mutant 6-031 strain preformed by Liu (2001).

The *cfs1* gene encodes a protein which is highly similar to the bacterial cyclopropane fatty acid synthases, a class of enzymes shown in prokaryotes and recently in a plant to convert membrane-bound unsaturated fatty acids into cyclopropane fatty acids (Liu 2001; Wang et al. 1992; Bao et al. 2002). In the bacteria, CFAs transfer a methylene-group derived from the methyl-group of *S*-adenosyl-*L*-methionine (SAM) across the carbon-carbon double bonds of membrane localized unsaturated fatty acids (UFAs) resulting in cyclopropane fatty acids (Grogan et al. 1997). In consequence of the cyclopropane fatty acid production, membrane properties alter. The fluidity of the membrane decreases and, with it, transport through the membrane (Chang et al. 1993; Wang et al. 1994; Grogan and Cronan 1997). This alteration of membrane properties is the basis of mediating stress resistance by the membrane. Bacterial strains which have high levels of CFAs survive in an acid shock milieu much more efficiently than strains which have low CFAs levels amongst the membrane-bound phospholipids (Chang et al. 1993; Wang et al. 1994; Grogan and Cronan 1997).

To proof the function of the *C. cinerea* protein Cfs1 in formation of cyclopropane fatty acid synthase, Loos (2001) inserted cDNA of the *C. cinerea* gene into the vector pET16b (Novagen) and

tested the construct in an *E. coli cfa* mutant lacking the bacterial enzyme CFA, which act as cyclopropane fatty acid synthase in wild type strains of this bacterium. Acid tolerance was found to be increased upon transformation but a suitable control by the *E. coli cfa* gene was lacking in the experiment of Loos (2001). In future, studies on function of the *cfs1* gene of *C. cinerea* in the bacterium need clearly to be done in comparison with the wildtype *cfa* gene of *E. coli,* utilizing gene transformation into the *cfa* mutant bacterium. Vector constructs being identical but in the bacterium to be expressed genes are needed for this experiment. Analogously to the *cfs1* cDNA in the pET-16b (Loos 2001), a vector pET-16b-*cfa* was therefore constructed (Fig. 1). From such comparable *E. coli* transformation study with the analogous vector constructs, we should be able to definitively deduce whether the *cfs1* gene of *C. cinerea* functions in the same way as the *cfa* wildtype gene of *E. coli*, with the hope that the results of such experiment will help us to get a better understanding on the functions of the Cfs1 protein in *C. cinerea*.

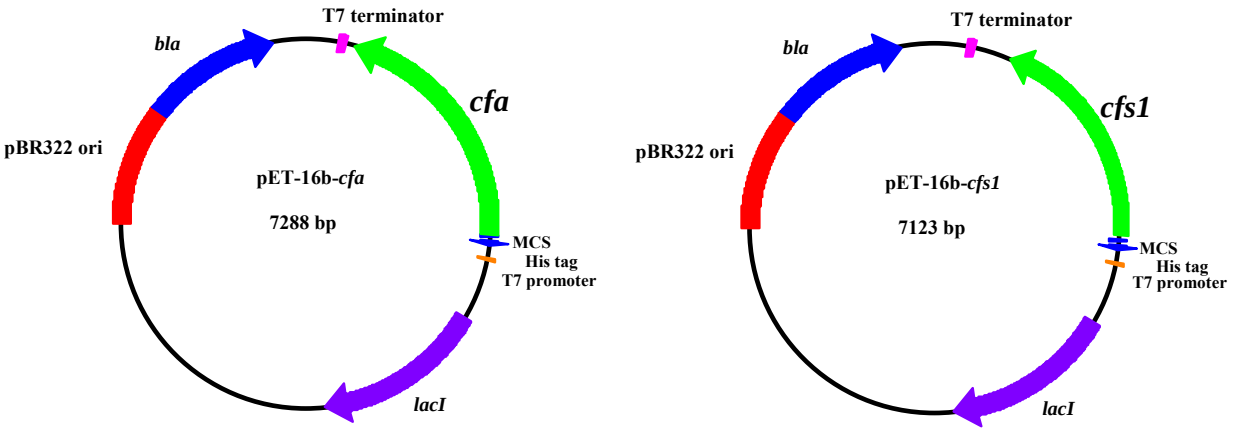


Figure 1. The structure of plasmid pET*cfa* (Srivilai, unpublished) and pET*cfs1* (Loos 2001). The *E. coli cfa* gene was subcloned as a 1575 bp B*am*HI fragment from plasmid pAYW19 (Grogan and Cronan 1997) into the site of the Novagen vector pET-16b.

10.5. The expression and functions of mating type genes in the basidiomycete *C. cinerea*: experiments with *C. cinerea* mating type genes

Mating type genes in higher basidiomycetes regulate mating of monokaryons with other compatible strains as well as clamp cell formation on the dikaryon and fruiting body development (Kües et al. 2002; Kothe 1999; Kronstad 2000). Tetrapolar species such as *C. cinerea* and *S. commune* have two distinct mating type loci called *A* and *B* that together determine the specific mating type of a monokaryon. Mating type is a genetically determined sexual incompatibility phenotype.

The *A* mating type genes encode the homeodomain transcription factors known as HD1 and HD2 proteins. For a compatible mating type interaction, a HD1 protein from one parental monokaryon has to interact with a HD2 protein from the other parental monokaryon. Compatible HD1 and HD2 protein combinations give in the dikaryon transcription factor complexes that regulate expression of clamp cell formation, synchronized nuclear division, production of asexual spores (oidia) and initiation of fruiting body development (Kües et al. 2002; Kües 2000).

In contrast, the *B* mating type genes encode pheromones and pheromone receptors. In a compatible mating interaction, a pheromone from a gene from one parental nucleus has to bind to a compatible pheromone receptor coming from a gene in the other parental nucleus. In the dikaryon, this reaction between compatible pheromones and pheromone receptors controls clamp cell fusion and steps in fruiting body development (Küse et al. 2002; Kües 2002; see above chapters 5 and 7 of this thesis). Mating type genes and their functions have been thoroughly studied in the past by transformation of subcloned genes into monokaryons of the same species that had however different mating type specificities as compared to the strains from which the cloned genes originated (e.g. for studies in *C. cinerea* see Pardo et al. 1996; Kües et al. 1992, 1994, 1998, 2002; O´Shea et al. 1998; Halsall et al. 2000).

Monokaryons transformed with compatible mating type genes of the same species perform morphological changes that are typical for the dikaryon. These changes in phenotype allow conclusions on which of the developmental processes in the dikaryon are regulated by the *A* mating type genes and which are regulated by the *B* mating type genes (Kües et al. 1992, 1994; O´Shea et al. 1998; Halsall et al. 2000). However, what can not be deduced from such studies is whether the products of the mating type genes have a function also in the monokaryon before mating. To obtain any knowledge in this direction, monokaryotic strains are required in which the mating type genes are inactivated or deleted. The NA2 strain (*Anull B6*) is a knock-out strain of the *A* mating type locus of *C. cinerea* obtained by deletion through gene replacement (Pardo 1995). This mutant is very useful for the discrimination of the individual *A* mating type genes (Pardo et al. 1996). The NA2 strain was used in this study to determine the possible function of individual *HD1* and individual *HD2* genes as well as to compare behaviour of HD1 and HD2 proteins coming from a same gene pair with the behaviour of HD1 and HD2 proteins coming from allelic gene pairs. Strain NA2 in morphology can not be distinguished from a normal monokaryon. It forms plain hyphae with simple septa. In the aerial mycelium, the strain produces abundant numbers of asexual spores (oidia) on oidiophores, both in the dark and in the light, similarly as does the normal monokaryon (Polak 1999).

In transformants of strain NA2, morphological changes at the hyphal septa were not observed as long as single *HD1* (*a1-3* or *a2-3*) or single *HD2* (*a2-1* or *a2-3*) or a pair of an *HD1*

gene and an *HD2* gene from the same mating type locus (*a1-3* and *a2-3*) were introduced together into the strain (chapter 6 of this thesis). This was different when a *HD1* gene and a *HD2* gene from allelic gene pairs (*a1-2* and *a2-1, a1-3* and *a2-1*, *a1-2* and *a2-3*) were introduced by co-transformation into strain NA2. Transformants of these pair of genes produced clamp cells at the hyphal septa (chapter 6 of this thesis), confirming an earlier report by Polak (1999) who tested before the compatible *A* mating type gene combination *a1-2* and *a2-1* in strain NA2. Transformants of such a pair of compatible *A* mating genes also produced few oidia in the dark and increased levels of oidia in the light, unlike an incompatible HD1 protein and HD2 protein combination coming from mating type genes of the same gene pair (*a1-3* and *a2-3*) (Polak 1999 and this thesis chapter 6).

Kertesz-Chaloupková and co-workers reported before that in *C. cinerea* i). normal monokaryons transformed with compatible *A* mating type genes (*A*on transformants), ii). homokaryons with a defect in the *A* mating type locus leading to constitutive activation of the *A* mating type pathway by a physical in frame fusion of an *HD2* gene to a normally incompatible *HD2* gene (*Amut B* homokaryons and *Amut Bmut* homokaryons) and iii). also dikaryons showed repression of oidia formation under dark growth conditions and that light overrides in all these strains this effect mediated by the *A* mating type genes. Therefore, from the experiments described in the literature as well as from the results of strain NA2 with the compatible *A* mating genes *a1-3* and *a2-1*, *a1-2* and *a2-1*, and *a1-2* and *a2-1,* respectively, in this study, it can be concluded that proteins from compatible genes from different haplotypes of the *A* mating type locus as well as proteins from mutated *HD2-HD1* fusion genes that mimic the compatible HD1 protein–HD2 protein interaction (further explanation in Kües et al. 1994) repress the asexual sporulation under dark conditions. Repression by a compatible HD1 protein – HD2 protein combination of asexual sporulation is counteracted by light (Kertesz-Chaloupková et al. 1998).

HD1 proteins contain a nuclear localization signal (NLS), and are therefore able to localize to the nucleus, unlike HD2 proteins (Spit et al. 1998). HD2 proteins are transferred to the nuclei by binding to compatible HD1 proteins (Spit et al. 1998). There is however the possibility that HD1 proteins also enter the nuclei without a bound HD2 protein in order to fulfil some regulatory function in the nucleus. Polak before transformed genes *a1-2* and *a2-1* also individually into strain NA2 and found that a tranformant of the *a1-2* gene had a 10-fold reduced oidia production in the dark as compared to the untransformed strain NA2 whereas the NA2 *a2-1* transformants behaved like a normal monokaryon with constitutive production of high number of spores (ca 10^9 spores/plate; Polak 1999). The data suggested that a HD1 protein alone might be able to also perform a repression of oidia production, although not as strong as a compatible HD1 protein - HD2 protein combination. Such an extra function of HD1 proteins independently from the HD2 proteins

would nicely repeat the regulatory pattern of homeodomain transcription factors encoded in the mating type loci of the ascomycete yeast *Saccharomyces cerevisiae.* In yeast, in a haploid cell of the α mating type, the α2 protein being homologous to the basidiomycete HD1 proteins (Kües and Casselton 1992) represses those genes that are determining the phenotype of the other mating type **a** (i.e. **a**-specific genes). After mating of two haploid cells of different mating type (α and **a**) in the diploid cell, where the α2 protein is present in addition to the **a1** protein, an HD2–type homeodomain transcription factors coming from the opposite mating type, the proteins α2 and **a1** heterodimerize in order to repress in the diploid cell all those genes that are specific to the haploid cells before mating (Herskowitz 1989).

In this study, more NA2 transformants of just *HD1* genes and just *HD2* genes were tested. However, a reduction a production of oidia in the dark and an increased production in light was found with some of the transformants of *HD1* genes (in particularly with gene *a1-3*) as well as with some of transformants of *HD2* genes (e.g. for some transformants of gene *a2-3*). From this study, it appears that a tenfold reduction in spore number might regularly occur in NA2 transformants, at least in parts independently of the mating type gene used for transformation. Generally, compared to monokaryons such as 218 and JV6, strain NA2 shows a somewhat irregular and slow growth (our unpublished observations). Possibly, a better *Anull* tester strain has to be constructed in the future by crosses of an *A* mating-type transformant of strain NA2 and one of the AmutBmut co-isogenic monokaryons created in this study to avoid any intrinsic irregularities in growth by the tester strain.

10.6. The expression and functions of mating type genes in the basidiomycete *C. cinerea*: experiments with foreign mating type genes

In 1998, Kües and co-workers studied in *A*on transformants of monokaryon 218 the function the *A* mating type genes of *C. cinerea*, and found that compatible *A* mating type genes possibly regulate the production of chlamydospores, hyphal knots and sclerotia. Hyphal knots on *A*on transformants further developed into fruiting body primordia, but they stopped to further develop at the developmental stage before karyogamy (Kües et al. 1998). Only when *A* mating type genes and *B* mating type genes of different specificity were simultaneously transformed into a monokaryon, fruiting body development could be completed (Kües et al. 2002). This showed that the *B* mating type genes are needed for later stages of development.

In this thesis, it was also attempted to express the *A* mating type genes from the distantly related higher basidiomycete *S. commune* into *C. cinerea* as well as the homologous *b* mating type gene of the heterobasidiomycete *U. maydis*. It was shown that also the foreign mating type genes of *U. maydis* and *S. commune* can trigger clamp cell formation in *C. cinerea* but only transiently in

some parts of the colonies of positive transformants (see chapter 9 for more details). Since single genes were transformed from *S. commune* and *U. maydis*, their proteins would be expected to interact with the endogenous *A* mating type protein of *C. cinerea* in regulation of development. An interaction by HD1 proteins from *A* mating type genes of *Coprinopsis scobicola* with HD2 protein of *C. cinerea* in regulating clamp cell formation in *C. scobicola* (Challen et al. 1993) and *C. cinerea* (Kües et al. 2001), respectively, had been demonstrated in the past by other authors (see chapter 5 for more details).

Further in this study, the *A* mating type genes of the bipolar species *C. disseminatus* isolated by Dr. Timothy Y. James (Department of Biology, Duke University, USA) were used to transform *C. cinerea* monokaryon 218. The results showed that the *A* mating type genes of *C. disseminatus* encode homeodomain transcription factors that are able to interact with the homeodomain transcription factors of *C. cinerea* monokaryon 218, since subsequently to transformation, clamp cell formation was observed (James et al. 2006). Thus, as it was shown before for *C. scobicola* (Challen et al. 1993), the study on *C. disseminatus* mating type genes showed that heterologous *A* mating type genes can be active in *C. cinerea*.

For the first time in this work, it was also demonstrated by transformation, that foreign pheromones and/or pheromone receptors can be functional in *C. cinerea* in inducing *B* regulated mycelial phenotypes (clamp cell fusion) as well as in controlling fruiting body development (chapter 7 of this thesis, James et al. 2006). What was most interesting in this study, the pheromones and/ or pheromone receptors genes from *C. disseminatus* do not have mating type function in this original host. Nevertheless, they may control clamp cell fusion and steps in fruiting body development also in *C*. *disseminatus*. It is possible that *B* mating type–like genes in *C. disseminatus* are constitutively active by interacting with each other (James et al. 2006) as it has been described in *C. cinerea* as a basis for the mutations in the *B* mating type locus leading to a constitutive activation of *B*-regulated functions (Olesnicky et al. 1998). If the *C. disseminatus* pheromones and pheromone receptors would indeed constitutively interact with each other, a direct interaction with the respective *C. cinerea* proteins would not be required in *C. cinerea* transformants. The results of the current study leave this question open. In order to solve this question in the future, isolated C. *disseminatus* pheromone genes and also isolated pheromone receptor genes would be needed to be transformed individually into *C. cinerea* to see whether under such conditions they have effects on development. Alternatively, a knock-out mutant of the whole *B* mating type locus could be transformed with the complete pheromone and pheromone receptor gene cluster from *C. disseminatus.* In case the products of the *C. disseminatus* genes interact with each other, *B*-regulated development should then be activated. There is however no *B* mating type locus -

knock-out strain available for such a study from *C. cinerea*, but from the other model species *S. commune* (Fowler et. 2001).

10.7. The *Ras* signaling pathway in *C. cinerea*

The *ras* gene of *C. cinerea* was first isolated by Ishibashi and his co-workers in 1993. (Ishibashi and Shishido 1993). Bottoli then isolated the *ras* allele from *C. cinerea* homokaryon AmutBmut (Swamy et al. 1984; Bottoli 2001). Ras proteins belong to a conserved family of GTP-binding proteins. In several eukaryotes it was shown that RAS acts as the molecular switch in various cellular signalling pathways and stimulates the adenylate cyclase in production of cAMP which in turn stimulates the cAMP–dependent protein kinase (PKA). Binding of cAMP to the four subunits of PKAs causes conformational changes of the subunits then by activating the catalytic functions PKA then can phosphorylate other proteins being parts of different signalling pathways (Leveleki et al. 2004). Our results from transformation experiments in *C. cinerea* with the constitutively activated *ras* ^{Val} showed that the Ras^{Val19} protein affected colony growth and the hyphal growth direction in both monokaryons and dikaryons, and it decreased in both types of colonies aerial mycelium. Lack of a defined growth direction indicates a defect in nutrient sensing. These results were consistent to previous reports showing results of other fungi which have been transformed by constitutively activated Ras proteins or have been blocked by mutation in the transition of the active GTP-form of RAS into the inactive GDP-bound form of RAS (Fortwendel et al. 2005; Kronstad et al. 1998; Schubert et al. 2006; Waugh et al. 2002; for details see chapter 8). In *S. cerevisiae,* Ras signaling causes an increase of intracellular levels of cAMP which regulates cell proliferation and carbon metabolism (Thevelein and Winde 1999; Alspaugh et al. 2000; for details see chapter 8). In other fungi, a relationship between cAMP production, nutrient sensing and the RAS pathway has also been shown (Kronstad et al. 1998).

Normally in the dikaryon of basidiomycetes, clamp cell formation occurs at the location where the mitosis is to be taken place. The clamp cell normally grows in the opposite direction to that of the hyphal tip growth. During mitotic divisions in the hyphal tip cell of a dikaryon, one of the two daughter nuclei of a parental nucleus migrates in the direction of the clamp cell and the other daughter nucleus migrates forward to the hyphal tip. The daughter nuclei of the other nucleus divide along the main hyphal axis. Septa are formed in between the two pairs of dividing daughter nuclei, one of which in between the clamp cell and the newly forming apical hyphal cell and the other within the foremost hyphal compartment separating it into the new apical and the new sub-apical cell. Subsequently, a peg is formed on the sub-apical cell close to the septum in order to fuse with the tip of clamp cell (Badalyan et al. 2004, Iwasa et al. 1998).

In *C. cinerea*, clamp cell formation was found to be influenced by the Ras^{Val19} protein. Our results showed an abnormality of clamp cell formation in dikaryons (218/*ras* ^{Val19} transformant X PS001-1 and FA2222/*ras* ^{Val19} transformant X PS001-1). The tips of abnormal unfused clamp cells were longer than that of normal clamp cells and most of the tips of abnormal clamp cells at *ras* ^{Val19} hyphae grew past the peg formed for clamp cell fusion at the sub-apical cells. Consequently, the clamp cells failed to fuse. The occurrence of the unfused clamp cells might be due to a lost signal that makes the clamp cells to recognize the respective pegs at the sub-apical cells. This signal is postulated to be the interaction of the pheromones and pheromone receptors which are encoded by compatible *B* mating type genes (Brown and Casselton 2001). Schubert and colleagues reported similar abnormal clamp cell phenotypes in dikaryons of *S. commune* carrying no *Gap1* gene regulatory the contribution of a RAS protein or a similar small GTPase also in *S. commune* (Schubert et al. 2006).

In *C. cinerea,* the Ras ^{Val19} protein also affected the fruiting body development by increase in the numbers of secondary hyphal knots, by increase in primordia formation, by decrease in the sizes of primordia and by alteration of the tissue structure of stipes and caps. Moreover, the Ras^{Val19} protein also negatively influenced the formation of basidiospores of *C. cinerea* in both quantity and quality. Again, similar observations were made in the *S. commune* dikaryon lacking the *GAP1* gene (Schubert et al. 2006).

In *C. cinerea* fruiting bodies of the *ras*^{Val19} dikaryon 218/*ras*^{Val19} transformant X PS001-1, meiosis in basidia was not disturbed. Thus, Ras signaling in *C. cinerea* is postmeiotically active in the formation of basidiospores (see chapter 8 of this thesis). This stage is very sensitive to genes in nutrients and quickly arrests development of basidospores when nutrients become suddenly available (Moore 1998). Furthermore, it is striking that both the defects in the clamp cell fusion and in early steps of fruiting (secondary hyphal knot formation, Kües et al. 2002) in disturbed Ras strains are *B*-mating type controlled phenotypes. Deletion of the GAP gene *sar* of a diploid *S. pombe* strain showed also severe sporulation defects (Wang et al. 1999). In this fungus, karyogamy and meiosis occurs directly before sporulation, in direct dependence of nutrients and of pheromones and pheromone receptors whose expression is controlled by the mating type genes (Hiscock and Kües 1999).

10.8. Gene expression in *C. cinerea* from *S. commune* hydrophobin gene promoters (*Sc3* and *Sc4*)

A kind of very interesting proteins known in filamentous fungi are the hydrophobias, which are secreted by filamentous fungi when leaving the aqueous environments for growing into the air. *S. commune* has at least 4 hydrophobin genes called *SC1*, *SC3*, *SC4* and *SC6* (Wessels et al. 1995).

The *SC3* gene is active in both the monokaryons and the dikaryons of *S. commune*. The *SC4* gene expresses in the dikaryon and at a higher level than the *SC1* and *SC6* genes during fruiting body development. The *SC4* gene is expressed highest at the time when the primordia are formed (Mulder and Wessels 1986).

Interestingly, using a laccase gene as a reporter (Kilaru et al. 2006), it was found that the *Sc3* promoter of *S. commune* was active in both the monokaryons and the dikaryons of *C. cinerea*. Therefore, the *Sc3* promoter can be used for a high-level gene expression system in *C. cinerea* (Kilaru et al. 2006; chapter 9 of this thesis). Expression from the *Sc3* promoter was also seen at a high level in *C. cinerea* fruiting body development. In contrasts, the *Sc4* promoters showed lower activity in the monokaryons than in the dikaryons and during the fruiting body development of *C. cinerea* (chapter 9 of this thesis). Thus, the control of *Sc3* and *Sc4* promoter activity in *C. cinerea* only partially resembled that in the native host *S. commune*.

10.9. General conclusions regarding mating type genes in basidiomycetes and control of expression of genes regulated by mating type genes

In this study, *A* mating type genes from *C. cinerea* and related mating type genes from other basidiomycetes have been analyzed in transformation of *C. cinerea* as well as genes that are either directly or indirectly regulated during growth and fruiting body by mating type genes. It was found that functions of mating type genes and of mating type-regulated genes in the heterologous host in some but not all cases were exactly the same as the functions of homologous genes coming from *C. cinerea* itself. Furthermore, genes from the foreign species *C. disseminatus* encoding pheromones and pheromone receptors were shown to react in *C. cinerea* as the native *B* mating type genes. From these experiments, it can be concluded that larger parts in developmental regulation within basidiomycetes are conserved. Therefore, the model fungus *C. cinerea* can be used to study the basic functions in development as a first understanding also for their basidiomycetes that are not or not as easily accessible by genetic techniques.

10.10. References

Alspaugh, J. A., L. M. Cavallo, J. R. Perfect, and J. Heiman, 2000 RAS 1 regulates filamentation mating and growth at high temperature of *Crytococcus neoformans*. Mol. Microbiol. **36:** 3206-3217.

Alspaugh, J. A., R. Pukkila-Worly, T. Harashima, L. M. Cavallo, D. Funnell *et al.*, 2002 Adenylyl cyclase functions downstream of the G protein Gpa1 and controls mating and phathogenicity of *Cryptococcus neoformans*. Eukaryot. Cell **1:** 75-84.

Arima, T., M. Yamamoto, A. Hirata, S. Kawano, and T. Kamada, 2004 The *eln3* gene involved in fruiting body morphogenesis of *Coprinus cinereus* encodes a putative membrane protein with a general glycosyltransferase domain. Fungal Genet. Biol. **41:** 805-812.

Badalyan, S. M., E. Polak, M. Aebi, and U. Kües, 2004 Role of peg formation in clamp cell fusion of homobasidiomycete fungi. J. Basic Microbiol. **44:** 167-177.

Banham, A. H., R. N. Asante-Owusu, B. Göttgens, S. A. J. Thompson, C. S. Kingsnorth *et al.*, 1995 An N-terminal dimerization domain permits homeodomain proteins to choose compatible partners and initiate sexual development in the mushroom *Coprinus cinereus*. Plant Cell **7:** 773-783.

Bao, X., J. J. Thelen, G. Bonaventura, and J. B. Ohlrogge, 2003 Characterization of cyclopropane fatty-acid synthase from *Sterculia foetida*. J. Biol. Chem. **278:** 12846-12853.

Bardwell, L., J. G. Cook, C. J. Lnouye, and J. Thorner, 1994 Signal propagation and regulation in the mating pheromone response pathway of the yeast *Saccharomyces cerevisiaevisiae*. Dev. Biol. **166:** 363-379.

Bottoli F. P. A., 2001 *Metabolic and Environmental Control of Development in Coprinus cinereus.* Ph.D thesis, Swiss Federal Institute of Technology Zurich, Zurich, Switzerland.

Brown, A. J., and L. A. Casselton, 2001 Mating type in mushrooms: increasing the chances but prolonging the affair. Trends Genet. **17:** 393-400.

Dix, N., and Webster J. 1995 *Fungal Ecology,* Ed. 2. Chapman and Hall, London.

Grogan, D. W., and J. E. Cronan, 1997 Cyclopropane ring formation in membrane lipids of bacteria. Microbiol. Mol. Biol. Rev. **61:** 429-441.

Griesser, B., 1992 *Mykosoziologie der Grauerlen- und Sanddorn-Auen (Alnetum incanae, Hippophaetum) am Hinterrhein (Domleschg, Graubbünden, Schweiz)*, Vol. 109. Veröff. Geobot. Inst. ETH, Stiftung Rübel.

Fortwendel, R. J., W. Zhao, R. Bhabhra, S. Park, D. Perlin *et al.*, 2005 A fungus-specific *ras* homolog contributes to the hyphal growth and virulence of *Aspergillus fumigatus*. Eukaryot Cell **4:** 1982-1982.

Fowler, T., M. F. Mitton, L. J. Vaillancourt, and C. A. Raper, 2001 Changes in mate recognition through alteration of pheromone and receptors in the multisexual mushroom fungus *Schizophylum commune*. Genetics **158:** 1491-1503.

Halsall, J. R., M. J. Milner, and L. A. Casselton, 2000 Three subfamilies of pheromone and receptor genes generate multiple *B* mating specificities in the mushroom *Coprinus cinereus*. Genetics **154:** 1115-1123.

Heitman, J., 2002 Mating type specific and nonspecific PAK kinases play shared and divergent roles in *Cryptococcus neoformans*. Eukaryot. Cell **1:** 257-272.

Herskowitz, I., J. Rine, and J. Strathen, 1992 Mating type determination and mating-type interconversion in *Saccharomyces cerevisiae*, pp. 583-656 in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression*, edited by E.W. Jones, J. R. Pringel and J. R. Broach. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Hiscock, S. J., and U. Kües, 1999 Cellular and molecular mechanisms of sexual incompatibility in plants and fungi. Int. Rev. Cytol. **193:** 165-295.

Ishibashi, O., and K. Shishido, 1993 Nucletotide sequence of a *ras* gene from the basidiomycete *Coprinus cinereus*. Gene **125:** 233-234.

Iwasa, M., S. Tanabe, and T. Kamada, 1998 The two nuclei in the dikaryon of the homobasidiomycete *Coprinus cinereus* change position after each conjugate division. Fungal Genet. Biol. **23:** 110-116.

James, T. Y., P. Srivilai, U. Kües, and R. Vilgalys, 2006 Evolution of the bipolar mating system of the mushroom *Coprinellus disseminatus* from its tetrapolar ancestors involves loss of mating-type-specific pheromone receptor function. Genetics **172:** 1877-1891.

Kamada, T., R. Kurita, and T. Takemaru, 1978 Effects of light on basidiocarp maturation in *Coprinus macrorhizus*. Plant Cell Physiol. **19:** 263-275.

Kanda, T., and T. Ishikawa, 1986 Isolation of recessive developmental mutants in *Coprinus cinereus*. J. Gen. Appl. Microbiol. **32:** 541-543.

Kanda, T., A. Goto, K. Sawa, H. Arakawa, Y. Yasuda *et al.*, 1989 Isolation and characterization of recessive sporeless mutants in the basidiomycete *Coprinus cinereus*. Mol. Gen. Genet. **216:** 526-529.

Kilaru, S., 2006 *Identification of Fungal Multi-Copper Oxidase Gene Families: Overexpression and Characterization of Coprinopsis cinerea Laccase for Applications in Biotechnology*. Ph.D. thesis, Georg-August University of Göttingen, Göttingen, Germany.

Kilaru, S., P. J. Hoegger, and U. Kües, 2006 The laccase multi-gene family in *Coprinopsis cinerea* has seventeen different members that divide into distinct subfamilies. Curr. Genet. **50:** 45-60.

Moore, D., 1981 Developmental genetics of *Coprinus cinereus*: Genetic evidence that carpophores and sclerotia share a common pathway of initiation. Curr. Genet. **3:** 145-150.

Moore, D., 1998 *Fungal Morphogeneis*. Cambridge University Press, Cambridge.

Kertesz-Chaloupková, K., P. J. Walser, J. D. Granado, M. Aebi, and U. Kües, 1998 Blue light overrides repression of asexual sporulation by mating type genes in the basidiomycete *Coprinus cinereus*. Fungal Genet. Biol. **23:** 95-109.

Kothe, E., 2001 Mating-type genes for basidiomycete strain improvement in mushroom farming. Appl. Microbiol. Biotechnol. **56:** 602-612.

Kronstad, J. W., and S. A. Leong, 1990 The *b* mating-type locus of *Ustilago maydis* contains variable and constant regions. Genes Dev. **4:** 1384-1395.

Kronstad, J. W., and C. Staben, 1997 Mating type in filamentous fungi. Annu. Rev. Genet. **31:** 245-276.

Kronstad, J., A. De Maria, D. Funnell, R. D. Laidlaw, N. Lee *et al.*, 1998 Signaling via cAMP in fungi: interconnections with mitogen-activated protein kinase pathways: Ach. Microbiol. **70:** 395-404.

Kües, U., 2000 Life history and developmental processes in the basidiomycete *Coprinus cinereus*. Microbiol. Mol. Biol. Rev. **64:** 316-353.

Kües, U., P. J. Walser, M. J. Klaus, and M. Aebi, 2002 Influence of activated *A* and *B* mating type pathways on developmental processes in the basidiomycete *Coprinus cinereus*. Mol. Gen. Genom. **268:** 262-271.

Kües, U., M. Künzler, A. P. F. Bottoli, P. J. Walser, J. D. Granado, *et al.*, 2004 Mushroom development in higher basidiomycetes; implications for human and animal health, pp. 431-470 in *Fungi in Human and Animal Health*, edited by R. K. S. Kushwaha. Scientific Publishers, Jodhpur, India.

Leveleki, L., M. Mahlert, B. Sandrock, and Bölker M, 2004 The PAK family kinase *Cla4* is required for budding and morphogenesis in *Ustilago maydis*. Mol. Microbiol. **54:** 396-406.

Leslie, J. F., 1981 Inbreeding for isogeneity by backcrossing to a fixed parent in haploid and diploid eukaryotes. Genet. Res. **37:** 239-252.

Loos, S., 2001.*Charakterisierung eines essentiellen Gens für die Fruchtkörperinitiation im Basidiomyzeten Coprinus cinerea*. Master thesis, ETH Zürich, Zurich, Switzerland.

Liu, Y., 2001 *Fruiting Body Initiation in the Basidiomycete Coprinus cinereus*. Ph.D. thesis, Swiss Federal Institute of Technology Zurich, Zurich, Switzerland.

Liu, Y., P. Srivilai, S. Loos, M. Aebi, and U. Kües, 2006 An essential gene for fruiting body initiation in the basidiomycete *Coprinopsis cinerea* is homologous to bacterial cyclopropane fatty acid synthase genes. Genetics **172:** 873-884.

Mulder, G. H., and J. G. H. Wessels, 1986 Molecular cloning of RNAs differentially expressed in monokaryons and dikaryons of *Schizophylum commune*. Exp. Mycol. **10:** 214-227.

Muraguchi, H., and T. Kamada, 1998 The *ich1* gene of the mushroom *Coprinus cinereus* is essential for pileus formation in fruiting. Development **125:** 3133-3141.

Muraguchi, H., and T. Kamada, 2000 A mutation in *eln2* gene encoding a cytochrom P450 of *Coprinus ciner*eus affects mushroom morphogenesis. Fungal Genet. Biol. **29:** 49-59.

Muraguchi, H., T. Takemaru, and T. Kamada, 1999 Isolation and characterization of developmental variants in fruiting using a homokaryotic fruiting strain of *Coprinus cinereus*. Mycoscience **40:** 227-235.

Muraguchi, H., Y. Ito, T. Kamada, and O. S. Yanagi, 2003 A linkage map of the basidiomycete *Coprinus cinereus* based on random amplified polymorphic DNAs and restriction fragment length polymorphisms. Fungal Genet. Biol. **240:** 93-102.

O'Shea, S. F., P. T. Chaure, J. R. Halsall, N. S. Olesnicky, A. Leibbrandt, *et al.*, 1998 A large pheromone and receptor gene complex determines multiple *B* mating type specificities in *Coprinus cinereus*. Genetics **148:** 1081-1090.

Pardo, E. H., 1995 *Organization of the A Mating Type Locus of Coprinus cinereus*. Ph.D. thesis, University of Oxford, Oxford, UK.

Pardo, E. H., S. F. O'Shea, and L. A. Casselton, 1996 Multiple versions of the *A* mating type locus of *Coprinus cinereus* are generated by three paralogous pairs of multiallelic homeobox genes. Genetics **144:** 87-94.

Peddireddi S., R. Velagapudi, P. J. Hoegger, A. Majcherczyk, A. Naumann *et al.*, 2005. Multiple hydrophobin genes in mushrooms in *Proceedings of the Genetics and Cellular Biology of Basidiomycetes VI.*, edited by A. G. Pissabaro and L. Ramírez. Spain Universidad Pública de Navarra, Pamplona, Spain, in press.

Pukkila, P. J., and C. Skrzynia, 1993 Frequent changes in the number of reiterated ribosomal RNA genes throughout the life cycle of the basidiomycete *Coprinus cinereus*. Genetics **133:** 203-211.

Pukkila, P. J., and B. C. Lu, 1985 Silver staining of meiotic chromosomes in the fungus *Coprinus cinereus*. Chromosoma **91:** 108-112.

Raper, J. R**.,** 1966 *Genetics of Sexuality in Higher Fungi*. Ronald Press, New York.

Redhead, S. A., R. Vilgalys, J. M. Moncalvo, J. Johnson, and J. S. Hopple, 2001 *Coprinus* Pers. and the disposition of *Coprinus species* sensu lato. Taxon. **50:** 203-241.

Riquelme, M., M. P Challen, L. A. Casselton, and A. J. Brown, 2005 The origin of multiple *B* mating specificities in *Coprinus cinereus*. Genetics **170:** 1105-1119.

Schubert, D., M. Raudaskoski, N. Knabe, and E. Kothe, 2006 Ras GTPase-activating protein gap1 of the homobasidiomycete *Schizophyllum commune* regulates hyphal growth orientation and sexual development. Eukaryot. Cell **5:** 683-695.

Srivilai, P., W. Chaiseana, and U. Kües, 2005 Genetic analysis of *Coprinosis cinerea* mutants with defects in fruiting body development in *Proceedings of the Genetics and Cellular Biology of Basidiomycetes VI*, edited by A. G. Pissabaro and L. Ramírez. Spain Universidad Pública de Navarra, Pamplona, Spain, in press.

Spit, A., R. H. Hyland, E. C. J. Mellor, and L. A. Casselton, 1998 A role for heterodimerization in nuclear localisation of a homeodomain protein. Proc. Natl. Acad. Sci. USA **95:** 6228-6233.

Swamy, S., I. Uno, and T. Ishikawa, 1984 Morphogenetic effects of mutations at the *A* and *B* incompatibility factors of *Coprinus cinereus*. J. Gen. Microbiol. **130:** 3219-3224.

Thevelein, J. M., and J. H. D. E. Wind, 1999 Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. Mol. Microbiol. **33:** 904-918.

Van Wetter M. A., H. A. B. Wösten, and J. Wessels, 2000 SC3 and SC4 hydrophobins have distinct roles in formation of aerial structures in dikaryons of *Schizophyllum commune*. Mol. Microbiol. **36:** 201-210.

Ulje, C. B., and C. Bas, 1991 Studies in *Coprinus*, 2. Subsection setulosi of section *Pseudocoprinus*. Persoonia **14:** 275-339.

Velagapudi, R., 2006 *Extacellular Matrix Proteins in Growth and Fruiting Body Development of Straw and Wood Degrading Basisidiomycetes*. Ph.D. thesis, Georg-August-University of Göttingen, Göttingen, Germany.

Wang, A. Y., D. W. Grogan, and J. E. Cronan, Jr., 1992 Cyclopropane fatty acid synthase of *Escherichia coli*: deduced amino acid sequence, purification, and studies of the enzyme active site. Biochemistry **3:** 11020-11028.

Walser, P. J., 1997 *Environmental Regulation of Asexual Sporulation and Fruit Body formation in the Basidiomycete Coprinus cinereus*. Master thesis, ETH Zurich, Zurich, Switzerland.

Schubert, D., M. Raudaskoski, N. Knabe, and E. Kothe, 2006 Ras GTPase-activating protein gap1 of the homobasidiomycete *Schizophyllum commune* regulates hyphal growth orientation and sexual development. Eukaryot. Cell **5:** 683-695.

Wang, P., C. B. Nichols, K. B. Lengeler, M. E. Cardenas, G. M. Cox *et al.*, 1992 Cyclopropane fatty acid synthase of *Escherichia coli*: deduced amino acid sequence, purification, and studies of the enzyme active site. Biochemistry **31:** 11020-11028.

Wessels, J. G. H., G. Mulder, and J. Springer, 1987 Expression of dikaryon–specific and non-specific mRNAs of *Schizophyllum commune* in relation to environmental conditions and fruiting. J. Gen. Microbiol. **133:** 2557-2561.

Curriculum Vitae

Name: Prayook

Family name: Srivilai

Date of birth: July, 14, 1973

Place of birth: Roi-Et, Thailand

Address: Department of Biology, Faculty of Science, Mahasarakham University
Tumbon Khamreing, Amphor Kantarawichai, Mahasarakham, 44150, Thailand

Office Phone: +66-43-754245 extension 1160 **Fax:** 66-38-75-4245

Home Phone: +66-43-897227

URL: http://biology.msu.ac.th/html/view.php?file=staff/prayook

E-mail addresses: psrivil@gwdg.de, prayooks@hotmail.com

EDUCATION BACKGROUND

Institution	Field of study	Date of Completion	Degree received
Kudlingvittayakhom School	Sciences-Mathematics	1988/06	Grade 6
Rongkham School	Sciences-Mathematics	1992/06	Grade 12
Mahasarakham University	Biology	1996/03	B.Sc.(Biology)
Chulalongkorn University	Genetics	1999/06	M.Sc.(Genetics)

SCHOLARSHIPS:

- University Development Commission (UDC) scholarship for M.Sc. study at Chulalongkorn University, the largest and oldest State University of Thailand
- Mahasarakham University Ph.D. scholarship for study at Georg-August-University of Goettingen, Germany

PUBLICATIONS:

Liu Y., **Srivilai P.,** Loos S., Aebi M., and Kües U. 2006. An essential gene for fruiting body initiation in the basidiomycete *Coprinopsis cinerea* is homologous to bacterial cyclopropane fatty acid synthase genes. Genetics 172: 873-884.

James T.Y., **Srivilai P.,** Kües U., Vilgalys, R. 2006. Evolution of the bipolar mating system of the mushroom *Coprinellus disseminatus* from its tetrapolar ancestors involves loss of mating-type-specific pheromone receptor function. Genetics 172: 1877-1891.

Srivilai P., Muraguchi M., and Kües, U. 2006. Construction of co-isogenic strains of *Coprinopsis cinerea* homokaryon *AmutBmut* with different wild-type mating type specificities. (to be submitted)

S**rivilai P.,** Chaisaena W., and Kües U. (2005). Genetic analysis of *Coprinopsis cinerea* mutants with defects in fruiting body development. In: "**VI Genetics and Cellular Biology of Basidiomycetes**" (AG Pisabarro and L. Ramirez, eds.), Universidad Publica de Navarra, Pamplona, Spain, pp. 177-190.

Srivilai P., James T. Y., Vilgalys R., Chaisaena W., and Kües U. (2005). Heterologous expression of mating type genes in basidiomycetes. In: **VI** "**Genetics and Cellular Biology of Basidiomycetes VI.**" (AG Pisabarro and L. Ramirez, eds.), Universidad Publica de Navarra, Pamplona, Spain, pp. 139-150.

Clergeot, P.-H., Ruprich-Robert, G., Liu, Y., Loos, S., **Srivilai, P.,** Velagapudi, R., Göbel, S., Künzler, M., Aebi, M. and Kües, U., (2003). Mutants in initiation of fruiting body development of the basidiomycetes *Coprinus cinereus*. Fungal Genetics Newsletter**,** Supplement-50, p. 68.

CONFERENCE CONTRIBUTIONS:

2006

Srivilai, P., Kilaru, S. and Kües, U. **Effects of a dominant constitutively activated *ras*-gene on vegetative and sexual development in *Coprinopsis cinerea*.** VAAM Jahrestagung 2006, Jena, 19-22 March 2006 (Poster).
Abstract: BIOspektrum, Tagungsband zur VAAM-Jahrestagung 2006, Jena, 19.-22. März 2006. p. 132.

Kües, U., Velagapudi, R., Pemmasani, J.K., Navarro-Gonzalez, M., Hoegger, P., Kilaru, S., **Srivilai, P.**, Majcherczysk, M., Peddireddi, S., and Chaisaena, W. **Analysis of multi-gene families with functions in fruiting body development of *Coprinopsis cinerea*.** VAAM Jahrestagung 2006, Jena, 19-22 March 2006 (Poster).
Abstract: BIOspektrum, Tagungsband zur VAAM-Jahrestagung 2006, Jena, 19.-22. März 2006. p. 132 (Poster).

Srivilai, P., Kilaru, S., Navarro-Gonzalez, M., Chaisaena, W. and Kües, U. **A constitutively activated Ras-GTPase alters mycelial growth in *Coprinopsis cinerea* and affects *B* mating type regulated phenotypes in dikaryons and fruiting body development:** 22nd European Fungal Genetics Conference on 9-11, March, 2006 at Vienna, Austria (Poster).
Abstract: Conference book: 22nd European Fungal Genetics Conference on 9-11, March, 2006 at Vienna, Austria.

2005

Srivilai P., Chaiseana W., and Kües, U. **Expression of mating type genes in heterologous basidiomycetes**. In Conference book: Molecular biology of fungi 7th VAAM-Conference ‘’Molekularbiologie der Pilze’’ 3.-7 September 2005, Bochum, Germany.
Abstract: Conference book: Molecular biology of fungi 7th VAAM-Conference ‘’Molekularbiologie der Pilze’’ 3.-7 September 2005, Bochum, Germany.

Srivilai P., Chaiseana W., and Kües, U. **Mutants of *Coprinopsis cinerea* with defects in fruiting body development.** In Conference book: Molecular biology of fungi 7th VAAM-Conference ‘’Molekularbiologie der Pilze’’ 3.-7. September 2005, Bochum, Germany.
Abstract: Conference book: Molecular biology of fungi 7th VAAM-Conference ‘’Molekularbiologie der Pilze’’ 3.-7 September 2005, Bochum, Germany.

Pemmasani J. K, Holzwarth F., **Srivilai P.,** Liu Y., Loos S., Aebi M., and Kües U. **An essential gene for fruiting body initiation in the basidiomycete *Coprinopsis cinerea* is homologous to bacterial cyclopropane fatty acid synthase genes.** In Conference book: Molecular biology of fungi 7th VAAM-Conference ‘’Molekularbiologie der Pilze’’ 3.-7 September 2005, Bochum, Germany.
Abstract: Conference book: Molecular biology of fungi 7th VAAM-Conference ‘’Molekularbiologie der Pilze’’ 3.-7 September 2005, Bochum, Germany.

Srivilai P., James T.Y., Vilgalys R., Chaisaena W., and Kües U. **Heterologous expression of mating type genes in basidiomycetes**. Genetics and Cellular Biology of Basidiomycetes VI. Pamplona, 3-6 June 2005, Spain (Poster).
Abstract: Congress VI Genetics and cellular Biology of Basidiomycetes, Pamplona, 3-6 Jun, Part II Posters (AG Pisabarro, ed)

Srivilai P., Chaiseana W., and Kües, U. (2005). **Genetic analysis of *Coprinopsis cinerea* mutants with defects in fruiting body development**. Genetics and Cellular Biology of Basidiomycetes VI. Pamplona, 3-6 June 2005, Spain (Poster).
Abstract: Congress VI Genetics and cellular Biology of Basidiomycetes, Pamplona, 3-6 Jun, Part II Posters (AG Pisabarro, ed)

Srivilai P., Chaisaena W., and Kües U. **Classical genetics in fruiting body development of *Coprinopsis cinerea***. 24th Fungal Genetics Conference 2005, Asilomar, USA (Poster).
Abstract: Fungal Genetic Newsletter 52.

2004

Velagapudi R., Kilaru S., Hoegger P.J., Dwivedi R.C., **Srivilai P**., Peddireddi S., Navarro-González M., Pemmasani L.K., Zomorrodi M., Lange K., Majcherczyk A., and Kües U., **Multi-gene families in the basidiomycete *Coprinopsis cinerea***. Gordon Research Conferences, 20-25 June 2004, Plymouth, USA (Poster).
(no abstract)

Navarro-González M. , **Srivilai P**. , Majcherczyk A., and Kües U. **Initiation of fruiting body development in the basidiomycete *Coprinopsis cinerea*,** VAAM 2004, Sonderausgabe, Braunschweig. March 28-31, 2004. Braunschweig, Germany (Poster). Abstract: Biospektrum Sonderausgabe, 2004, ISSN-0947-0867.
Abstract: in BIOspektrum Sonderausgabe 2004, p.154.

Srivilai P., James T.Y., Vilgalys R., and Kües U. **Mating type genes in basidiomycetes**, 2nd International *Ustilago maydis* Meeting, 25-29 August 2004, Rauischholzhausen, Germany (Poster), and VAAM Workshop, Stoffwechsel der Pilze, Fachhochschule Lausitz 16-18 September 2004, Senftenberg, German (Poster).
Abstract: in conference book-Poster Session II Abstract.

2003

Göbel S., Clergeot P.H., Ruprich-Robert G., Liu Y., Loos S., **Srivilai P.,** Velagapudi R., Künzler M., Aebi M., and Kües U. **Mutants in initiation of fruiting body development of the basidiomycete *Coprinus cinereus***. In: Conference book: Molecular biology of fungi 6th VAAM-Conference ‘’Molekularbiologie der Pilze’’ 3-5 September 2003, Goettingen, Germany.
Abstract: Conference book: Molecular biology of fungi. 6th VAAM-conference
“Molekularbiologie der Pilze 3-5 September 2003“ Goettingen (U. Kües, ed.), Wissenchaftlicher Fachverlag Dr. Peter Fleck, Langgöns (Niederkleen), Germany, p.110.

Srivilai P., Liu Y., Loos S., Göbel S., Aebi M., and Kües U. **A fruiting-defective mutant of the basidiomycete *Coprinus cinereus* is blocked in secondary hyphal knot formation by a defect in a cyclopropane fatty acid synthase gene.** : Conference book: Molecular biology of fungi 6th VAAM-Conference ''Molekularbiologie der Pilze'' 3-5 September 2003, Goettingen, Germany.
Absract: Conference book: Molecular biology of fungi 6th VAAM-Conference ''Molekularbiologie der pilze'' 3.-5 September 2003, Goettingen, Germany (U. Kües, ed.), Wissenchaftlicher Fachverlag Dr. Peter Fleck, Langgöns (Niederkleen), Germany, p.111.

Clergeot P.H., Ruprich-Robert G., Liu Y., Loos S., **Srivilai P.,** Velagapudi R., Göbel S., Künzler M., Aebi M., and Kües U. (2003). **Mutants in initiation of fruiting body development of the basidiomycete *Coprinus cinereus*.** 22nd Fungal Genetics Conference on 18-23 March 2003 at Pacific Grove, California, USA (Poster).
Abstract: Fungal genetics Newsletter 50.

ORAL PRESENTATIONS:

Molecular analysis of genes in initiation of fruiting body development in basidiomycetes (*Coprinosis cinerea*) 6 May 2006. At Departments of Molecular Genetics and Microbiology (MGM), Pharmacology and Cancer Biology, and Medicine, Duke University Medical Center, Duke University, Durham, North Carolina, USA.

Molecular analysis of genes in initiation fruiting body development in basidiomycetes (*Coprinosis cinerea*) (PhD work) 11 Jan 2006. In the seminar of the PhD program Wood Biology and Technology at Georg-August University of Goettingen, Germany.

Expression of mating type genes and heterologous in heterologous basidiomycetes. 3-7 September 2005. At the 'Molecular biology of fungi 7th VAAM-Conference', Bochum, Germany.

Analysis of a cyclopropane fatty acid synthase (*cfs1*) gene in fruiting body development in *Coprinopsis cinerea*. 7 June 2005. In the Botanical Seminar at the Institute of Forest Botany, Georg-August University of Goettingen, Germany.

Classical genetics in *Coprinopsis cinerea*. 5 January 2004. In the Botanical Seminar at Institute of Forest Botany, Georg-August University of Goettingen, Germany.

Protein structure and Protein data bank. 14 July 2004. In the seminar of the PhD program Wood Biology and Technology at Georg-August-University of Goettingen, Germany.

Gene transformations in plant. 3 February 2003**.** In the seminar of the PhD program Wood Biology and Technology at Georg-August University of Goettingen, Germany.

Genes in initiation a fruiting body in basidiomycetes *Coprinopsis cinerea*. 14 May 2002. In Ph.D program at Georg-August University of Goettingen, Germany.